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Impacts of genotypic variations in sulfur distribution and branching characteristics on nitrogen efficiency of oilseed rape (*Brassica napus* L.)

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I don´t do drugs. I set plants on fire and breathe.

Albert Einstein

(1879-1955)

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Contents

Chapter 1

General introduction

1. General introduction

Oilseed rape (*Brassica napus* L.) is an important agricultural crop that represents a major renewable resource for human food (Malagoli *et al.*, 2005), animal feed (Schjoerring *et al.*, 1995) and numerous non-food uses (e.g. bio-fuel, lubricants, high added-value products derived from green chemistry; Girondé *et al.*, 2015). Also cultivation of this crop is valuable for diversifying the cereal-dominated crop-rotations (Ulas *et al.*, 2013) and for supersessing soil-borne pathogens (Lee *et al.*, 2014).Therefore, many EU countries have a great interest in oilseed rape production. The EU (27) is by far the world's largest producer with about 20 Mt per year (Oil World, 2012). Among the EU countries, Germany and France represent the leading ones in oilseed rape production with sowing area of 1.46 and 1.44 Mha in 2013, respectively (Carré and Pouzet, 2014).

Over the last 20 years, oilseed rape has become the second most important oleaginous crop worldwide due to a 2.4-fold increase in seed production between 1992 and 2012 (Carré and Pouzet, 2014). However, the rapid yield increase during the last decades is not simply owing to breeding of high-potential cultivars or sophisticated production engineering (Rathke *et al.*, 2006). Other than these practices, mineral nitrogen (N) fertilization plays a crucial role. As a heavy user of N, oilseed rape crops usually need high rates of N fertilizer to obtain maximum seed yield (Shepherd and SylvesterBradley, 1996; Bilsborrow *et al.*, 1993; Schjoerring *et al.*, 1995). Despite the high N demand, the N efficiency of winter oilseed rape has been found relatively poor (Aufhammer *et al.*, 1994; Rossato *et al.*, 2001; Schjoerring *et al.*, 1995). Due to a low N uptake from the soil and an incomplete N retranslocation from the vegetative biomass into the seeds, usually only 50% or less of applied fertilisation is recovered in the harvested seeds (Aufhammer *et al.*, 1994; Taylor *et al.*, 1991; Schjoerring *et al.*, 1995), leaving high soil mineral N contents and high N amounts in crop residues in the field

(Aufhammer *et al.*, 1994; Lickfett *et al.*, 2001). As a consequence, oilseed rape is characterized with higher N balance surpluses than other crops (Gäth, 1997), which increases the risk of environmental quality as well as the detriment of the economic return (Rathke *et al.*, 2006). In this context, the implementation of the EU Nitrate Directive in Germany restricts N balance surpluses to 60 kg N ha^{-1} averaged over 3 years, starting in 2009 on the farm level, which is, however, difficult to achieve, when oilseed rape is included in the crop rotation (Henke *et al.*, 2009).

1.1 Breeding for N efficient cultivars

A main approch is to reduce N fertiliser input and meanwhile to avoid severe yield penalties, which might be achieved by development and selection of oilseed rape cultivars that can efficiently use the available N. N efficiency of a genotype is defined as the ability to produce a above average yield under soil conditions that are N-limiting for a standard genotype (Graham, 1984). N-efficient genotypes are therefore selected under low N supply. On the other hand, the realisation of a high yield potential with increasing fertilizer rates might also play a role in reducing N balance surpluses, since high seed yields can enhance N removal from the field. Cultivars with the capacity to increase yield when N supply is increased, are called N responders (Gerloff, 1977). Since N responsive cultivars are not necessarily N-efficient and vice versa (Blair, 1993), N efficiency and N responsiveness have to be investigated separately.

To facilitate and accelerate the selection of N-efficient genotypes in the breeding process, a comprehensive understanding of the underlying physiological mechanisms is advantageous. Generally, N efficiency can be seperated into two components: N uptake and N utilisation efficiency (seed dry matter yield/total N uptake; Moll *et al.*, 1982). Previous studies have highlighted that N-efficient oilseed rape cultivars are characterized in the first place by high N uptake efficiency under N limiting conditions (Berry *et al.*, 2010; Schulte auf'm Erley *et al.*,

2011; Kessel *et al.*, 2012). But N utilisation efficiency becomes more important for tracing genotypic variation in seed yield with inceasing N supply (Schulte auf'm Erley *et al.*, 2011; Kessel *et al.*, 2012).

N uptake is related to the size and effectiveness of the root system (Jackson *et al.*, 1986), while N utilisation efficiency is associated with the N distribution in the plant and the ability to assimilate and convert CO2 into grain carbohydrates (Sattelmacher *et al.*, 1994). The conversion of absorbed N into biomass has been found rather efficient in oilseed rape when compared to wheat (Dreccer *et al.*, 2000). The importance of N remobilisation to achieve high yields, however, is less clear. Although no significant genotypic correlations between N harvest index (seed N/total N uptake) and yield have been proven at either low or high N supply (Schulte auf'm Erley *et al.*, 2011), it can not be excluded that variation in N remobilisation plays a role in yield formation, since more than 70 % of pod N is derived from N remobilisation (Malagoli *et al.*, 2005; Gombert *et al.*, 2010).

1.2 Reproductive growth phase is critical for N efficiency

To facilitate the breeding process of such N-efficient cultivars, many studies on genotypic variation in N efficiency of oilseed rape have been carried out to identify the secondary plant traits contributing to N efficiency (Erley *et al.*, 2011; Erley *et al.*, 2007; Kessel *et al.*, 2012; Ulas *et al.*, 2013; Girondé *et al.*, 2015; Nyikako *et al.*, 2014). Two possible ideotypes of N-efficient cultivars have been hypothesized previously (Wiesler *et al*, 2001): an 'improved traditional ideotype' with vigorous growth and high N uptake during vegetative growth and efficient N remobilisation into seeds during reproductive growth and an 'alternative ideotype' with relatively slow growth and N uptake rates during vegetative stage, which, however, continue during reproductive growth. Various long-term field, pot and nutrient solution experiments (Wiesler *et al*, 2001, Behrens, 2002; Ulas, 2010, Ulas *et al*, 2013) suggest that N-efficient rapeseed cultivars can be assigned to the 'alternative ideotype', because positive

correlations of seed yield with N uptake during reproductive growth under conditions of low N supply have been found, but not with vegetative N uptake. Balint and Rengel (2008) also found that shoot dry weight, N uptake and N utilization efficiency at the vegetative growth stage were not indictive for N efficiency parameters at maturity, while other studies underlined that seed set (Berry *et al.*, 2010) and N uptake after flowering (Schulte auf'm Erley *et al.*, 2011) were related to high N efficiency. Since studies on this topic have mainly highlighted the importance of the reproductive growth for a high yield under conditions of low N supply, and a minor importance of the vegetative growth, the processes decisive for reproductive growth should receive more attention in order to identify the decisive plant traits for N efficiency. Previous studies have revealed that reproductive development of oilseed rape can be influenced by various genetic and environmental factors (Bosac *et al.*, 1993; Frenck *et al.*, 2013; Chen *et al.*, 2014; Gomez and Miralles, 2011; Gul and Ahmad, 2007). This thesis covers two possible aspects, i.e genotypic variations in sulfur distribution and in branching traits. The main focuses are aiming at the influences of these genotypic variations on seed yield and N efficiency.

1.3 Effect of genotypic variation in sulfur distribution on N efficiency

Apart from N, oilseed rape also has a high demand for sulfur (S) (Holmes, 1980), because this crop produces seeds with a high yield of protein with relatively large quantities of S-containing amino acids (Zhao *et al.*, 1997), and the plants require S for the synthesis of glucosinolates (Chew, 1988). N and S are both involved in amino acid and protein synthesis. They are tightly linked during the growth cycle, i.e N and S requirement and metabolism in plants are closely interrelated (Reuveny *et al.*, 1980; Janzen and Bettany, 1984; Fismes *et al.*, 2000). While N nutrition usually improve both, vegetative and reproductive growth, S specifically enhances the reproductive growth in oilseed rape (Janzen and Bettany, 1984; McGrath and Zhao, 1996). A high S nutrition promotes the formation of yield components. Especially pod abortion is prevented with sufficient S supply (Zhao *et al.*, 1993; Fismes *et al.*,

2000), but also the number of seeds per pod and thousand kernel weight can be improved by enhanced S supply (Amanullah *et al.*, 2011). A sufficient S supply of pods and seeds might therefore be a decisive factor for a high N efficiency. In particular, mineral S availability between stem extension and beginning of flowering is a determinant for seed filling processes and seed quality (Dubousset *et al.*, 2010), whereas little S uptake is generally observed during pod filling (McGrath and Zhao, 1996; Postma *et al.*, 1999). It seems thus that redistribution of S taken up before flowering would play a crucial role in seed filling and contribute to the maintenance of seed yield. However, genotypic relationship between N and S redistribution during seed filling and the influence of N and S interplay in these processes on N efficiency remains to be elucidated.

In this context, a series of experiments have been conducted in this study with the hypothesis that S remobilization will be depressed by limiting N supply (Chapter 2) and that cultivars with superior S remobilization can have promoted reproductive growth and thus higher seed yield and N efficiency (Chapter 3, 4). Firstly, a hydroponic experiment was conducted using four commercial oilseed rape cultivars grown under both low and high N conditions to check genotypic variations in S metabolism (Chapter 2). As substantial cultivar difference was evident in S metabolism only at low N supply, the following four hydroponic experiments including a set of 22 cultivars (13 double low and 10 high glucosinolate cultivars) were carried out under low N condition in order to find out clues as to how genotypic variations in leaf sulfur metabolites can lead to difference in S distribution and leaf N remobilization (Chapter 3). Thereafter, a set of DL and HG cultivars that were found to vary in S distribution were grown at low and high N supply in three-location field experiments. It was explored if genotypic variation in S distribution can be related to yield and harvest index and might thus present a valuable plant trait to improve yield and N efficiency of oilseed rape (Chapter 4).

1.4 Effect of genotypic variation in branching on N efficiency

Plant branching is a key process in the yield elaboration of winter oilseed rape (Pinet *et al.*, 2015). Variations in branching characteristic can lead to differences in seed yield and yield components (Chauhan *et al.*, 1987). Improved branching has been suggested as one mean to improve yield since the numbers of primary and secondary branches have a significant and positive correlation with seed yield (Katiyar and Singh, 1974; Ma *et al.*, 2014). On the other hand, an improved branching, however, may lead to greater retention of both dry matter and N in vegetative structure (Chauhan *et al.*, 1987), and thus poor harvest index and N harvest index, which are frequently yield-limiting factors in this crop. In particular, the low-ordered, i.e. late-flowering branches generally produce fewer flowers and pods than the higher-ordered ones. Also buds, flowers and pods on these branches are lost more quickly (Tayo and Morgan, 1979). Consequently these low productive or non-productive branches can form 'parasitic' sinks for assimilates. In contrast, de-branching of the basal portion can induce compensatory growth in higher-order branches mainly through restoration of the number of pods and thus beneficial for total yield (Chauhan *et al.*, 1987; Tommey and Evans, 1992). In these studies, however, plant N efficiency was not described.

Apart from the apical-basal sequence, secondary branches are also generally later flowering than the primary ones. Compared with the primary branches, the secondary ones are less productive (Chauhan *et al.*, 1987; Pinet *et al.*, 2015). But it is unknown whether genotypic variation in assimilate investment in these primary/secondary inflorescences can influence seed yield. Therefore, a pot experiment including three oilseed rape cultivars differing in branching characteristics was conducted, in order to find out clues how branching differences can affect seed yield and N efficiency (Chapter 5).

1.5 Objectives

On the basis of 'reproductive growth determinant' observations, the current study hypothesizes that genotypic variations in S distribution and branching type influence N efficiency of oilseed rape cultivars. This thesis provides a comprehensive analysis of genotypic variations in N and S metabolism, plant S distribution, N fluxes in various organs as well as N efficiency traits, with the following objectives:

(1) To investigate if cultivars differ in leaf N and S remobilization (Chapter 2)

(2) To determine genotypic variation in the involved S metabolites that can lead to a difference in leaf N remobilization as well as S distribution to developing organs (Chapter 3).

(3) To check if the observed genotypic variation in S distribution (Chapter 3) can be linked to yield and harvest index and thus act as a valuable plant trait to improve yield and N efficiency of oilseed rape (Chapter 4).

(4) To reveal how branching characteristics influence rapeseed yield and N efficiency (Chapter 5).

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Chapter 2

Sulfur metabolism of winter oilseed rape cultivars is differentially affected by N deficiency

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Sulfur metabolism of winter oilseed rape cultivars is differentially affected by N deficiency

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3 tables

1 figure

Running title: Sulfur metabolism of rape cultivars under N deficiency

Key words: nitrogen efficiency; genotypic variation; sulfur remobilization; sulfate accumulation; *Brassica napus* L.

Abstract

Genotypic variation in nitrogen (N) efficiency of oilseed rape is mainly related to differences in reproductive growth. These differences might be related to a genotypic variation in sulfur (S) availability for pod and seed set. In the current study, it was hypothesized that a physiological S deficiency in growing plant parts might be induced under N-limiting conditions due to a high sulfate accumulation in mature leaves which is poorly remobilized. This hypothesis was tested by comparing leaf S remobilization from senescing leaves under high and low N supply in four oilseed rape cultivars. The cultivars were grown in hydroponics in the greenhouse and leaf senescence was induced by shading. Low N conditions did not lead to the proposed relative increase of sulfate in mature leaves. Total S remobilization from the leaves was higher under low N than under high N conditions. The proposed physiological S deficiency in young plant parts might therefore not be more probable under low than under high N conditions. However, genotypic variation in S uptake and remobilization was found under N-limiting conditions only and might therefore contribute to genotypic variation in reproductive growth.

1 Introduction

In European agriculture a serious problem in the cultivation of winter oilseed rape is the high nitrogen (N) balance surplus which has a negative impact on the environment and decreases the economic return. To reduce the high N balance surplus without yield penalty, improvement of soil- and fertilizer-N management and the breeding and cultivation of N-efficient cultivars are necessary (Rathke *et al*. 2006; Sylvester-Bradley and Kindred 2009). Identification of plant characteristics correlating with N efficiency could facilitate the selection process of N-efficient cultivars. The physiological background of an improved genotypic N efficiency, however, has not been clarified yet. Studies performed on the topic until now have mainly highlighted the importance of the reproductive growth phase for a high yield at low N supply, and a minor importance of the vegetative growth phase. Shoot dry weight, N uptake and N utilization efficiency at the vegetative growth stage were not indicative for N efficiency parameters at maturity (Balint and Rengel, 2008). In particular, seed set (Berry *et al*., 2010) and N uptake after flowering (Schulte auf'm Erley *et al*., 2011) were related to high N efficiency. These results indicate that the processes decisive for reproductive growth should receive more attention in order to identify the decisive plant traits for N efficiency.

Yield reductions in oilseed rape due to N limitation were not primarily caused by the decrease in carbon (C) availability during reproductive growth (Dreccer *et al*., 2000). Instead, a sink limitation seems to occur at low N. Apart from overall assimilate availability pod and seed set seem to depend particularly on the availability of N assimilates (Schjoerring *et al*., 1995; Diepenbrock, 2000). It has not been proven yet, however, which assimilates in detail are decisive. While C and N assimilates usually improve both, vegetative and reproductive growth, sulfur (S) supply specifically enhances the reproductive growth in oilseed rape, (Janzen and Bettany, 1984; McGrath and Zhao, 1996). A high S nutrition promotes the ion of yield components. Especially pod abortion is prevented with sufficient S supply (Zhao *et al*.,

1993; Fismes *et al*., 2000), but also the number of seeds per pod and thousand kernel weight can be improved by enhanced S supply (Amanullah *et al*., 2011). A sufficient S supply of pods and seeds might therefore be a decisive factor for a high N efficiency. Due to the strong interrelations in the plant N and S metabolism, there are several reasons to expect that the S metabolism could be impaired under N-limiting conditions:

Sulfate uptake is de-repressed by the presence of *O*-acetylserine (OAS) which is a precursor of cysteine and a key substance in the interplay between N and S metabolism (Maruyama-Nakashita *et al*., 2004). Under N limitation, OAS concentration is lowered (Kim *et al*., 1999), and therefore S uptake can be downregulated (Buchner *et al*., 2004). A high sulfur accumulation will therefore not occur at N limitation.

Sulfur assimilation is as well regulated by the N availability, presumably via OAS (Kopriva and Rennenberg, 2004). OAS thereby downregulates the gene expressions of several enzymes in sulfur assimilation pathway, especially adenosine phosphosulphate reductase (APR), which catalyzes the most limiting step in the assimilation (Davidian and Kopriva, 2010). Higher sulfate accumulation under N limitation is therefore likely and has also been found under low N supply and high S supply in field experiments (Janzen and Bettany, 1984).

A high sulfate accumulation can generally be found in oilseed rape (McGrath and Zhao, 1996) and was even proposed to cause the low S efficiency of this crop (McGrath and Zhao, 1996; Hawkesford, 2000). Although accumulated sulfate is an important source for S remobilization during high S demand (Sunarpi and Anderson, 1997a; Hawkesford, 2000), sulfate remobilization in oilseed rape was found to be very slow and might therefore be insufficient to fulfill the demand (Blake-Kalff *et al*., 1998; Hawkesford, 2000), 2000). The amounts of glucosinolates and glutathione in oilseed rape, on the other hand, were too low to significantly contribute to S remobilization (Blake-Kalff *et al*., 1998). An insufficient S remobilization

might therefore occur under conditions of a relatively high sulfate accumulation, which can be the case under N limitation.

Although there has recently been considerable effort to better characterize S remobilization in oilseed rape (Dubousset *et al*., 2009, 2010; Abdallah *et al*., 2010, 2011), almost all studies were focused on a comparison between sufficient S and S-starvation conditions. Interactions with low N supply were considered, but only in combination with low S supply (Sunarpi and Anderson, 1997b; Dubousset *et al*., 2009; Abdallah *et al*., 2010). Under conditions of low S supply to the roots, sulfate can be more easily transported from the vacuoles by enhanced expressions of tonoplastic sulfate transporters (Dubousset *et al*., 2009), but it is not known if S deficiency in growing plant parts can induce the same effect. To our knowledge the only study which investigated the effects of low N supply on S remobilization at sufficient S supply to the roots was the study of Blake-Kalff *et al*. (1998). Here, it was shown that sulfate remobilization was triggered by low S, but hardly by low N supply.

The hypothesis of the presented study was therefore that under N-limiting conditions S accumulation in form of sulfate in mature leaves is enhanced. The sulfate-S stored in mature leaves is then insufficiently remobilized during reproductive growth, thereby decreasing the formation of reproductive structures and thus yield and N efficiency. In order to test the underlying assumptions, a hydroponic experiment was performed using oilseed rape cultivars grown at low and high N supply and sufficient S. S uptake, sulfate accumulation and S remobilization from senescing leaves were compared between low and high N supply to identify the steps in S metabolism which are most strongly impaired by N limitation. In addition, four commercial winter oilseed rape cultivars were included into the study to evaluate genotypic variation in these traits with possible implications for N efficiency.

2 Material and Methods

2.1 Experimental setup

A hydroponic experiment was performed in the greenhouse of the Institute of Plant Nutrition and Soil Science, University of Kiel, Germany from 5 October to 12 November 2013. Four commercial winter oilseed rape cultivars Aragon, Alaska, Savannah and Expert were selected for the study due to their differences in S uptake and S assimilation in a previous hydroponic experiment (data not shown). Two N rates were established: 0.5 mM N (low N) and 2.0 mM N (high N). Seeds were germinated between filter paper and moistened with tap water. After germination, four seedlings from each cultivar were transplanted into continuously aerated nutrient solution in a 10 L plastic pot and thinned to two seedlings per pot after 8 days. Aerated nutrient solution was at first supplied at 25% strength and then increased to 50% strength on day 3. From day 5 on, plants received the full nutrient solution with the following composition: 1000 µM KCl, 200 µM $CaCl_2$, 200 µM K_2HPO_4, 75 µM KH_2PO_4, 300 µM $MgSO_4$, 10 µM H_3BO_3, 2 µM $MnSO_4$, 0.5 µM ZnSO4, 0.2 µM $CuSO_4$, 0.05 µM $(NH_4)_2Mo_7O_{24}$ and 60 µM Fe-EDTA. N concentrations for low and high N treatments were set as 0.5 mM N (200 µM $Ca(NO_3)_2$ and 50 µM NH_4NO_3) and 2.0 mM N (800 µM $Ca(NO_3)_2$ and 200 µM NH_4NO_3). The nutrient solution was replaced twice a week to maintain the set concentrations. 24 days after transplanting, the third leaf (leaf 3) counted from the base of the plants was fully expanded for all plants. Leaf 3 from one plant in each pot was covered with aluminium film to induce leaf senescence and nitrogen and sulfur remobilization, while leaf 3 from the other plant was left untreated and used for comparison. The harvest was performed 38 days after transplanting when the covered leaf was shed from the plant. The experiment was completely randomized with 4 replicates.

2.2 Measurements

Harvested plants were separated into roots, leaf 3 and the rest shoot, and then oven dried to a constant weight for dry weight (DW) determination. Afterwards, plant samples were ground into fine powder and total N and S concentrations were determined according to the Dumas combustion method using a CNS elemental analyzer (Flash EA 1112 NCS, Thermo Fisher Scientific, Waltham, MA, USA).

Sulfate concentration was determined by ion-chromatography. For extraction, 1.5 mL de-ionized water was added to 0.03 g of dried plant material and promptly transferred to a boiling water bath for 5 min. After incubation on ice for at least half an hour, samples were centrifuged and proteins were removed from the solution by extraction with chloroform. The supernatant was filtered on C18-columns (Strata 8B-S001-DAK) for further purification. If necessary, extracted solutions were diluted prior to analysis by ion-chromatography (ICS 2500, Dionex, Sunnyvale, CA, USA).

2.3 Calculations and statistical analyses

Apparent N remobilization efficiency (ANRE) was estimated with the following equation:

$$\mathrm{ANRE} = \frac{[\mathrm{N}]_{\mathrm{Mat..}} - [\mathrm{N}]_{\mathrm{Sen.}}}{[\mathrm{N}]_{\mathrm{Mat.}}} \times 100\%$$

with $[N]_{Mat.}$ and $[N]_{Sen.}$ presenting the N contents (mg leaf^{-1}) of the mature and senescent leaf 3, respectively. Apparent S remobilization efficiency (ASRE) and apparent biomass remobilization efficiency (ABRE) were calculated accordingly.

Statistical tests were performed with R 3.0.2. Three-way ANOVA was used to test N rate, cultivar and covering and their interaction effects on leaf DW, N, S, and sulfate-S concentration, N:S ratio and sulfate-S:S ratio. Since no covering effects were found on total shoot parameters, the data for the shoots was summarized and a two-way ANOVA was performed to check N rate, cultivar and N rate × cultivar interaction effects. The two-way

ANOVA was used for shoot DW, N and S uptake, shoot N, S and sulfate-S concentrations, N:S ratio, sulfate-S:S ratio, leaf ABRE, ANRE and ASRE. To check for cultivar differences within N rates, multiple comparisons were performed using the Tukey test in the R package 'multcomp'.

3 Results

3.1 Shoot parameters

Low N supply significantly reduced shoot dry weight and N uptake (Table 1), especially for cv Expert, which had the lowest shoot DW and N uptake at low N supply. Moreover, shoot N concentration was also decreased significantly by low N, with cv Expert having a comparatively higher shoot N concentration than the other cultivars. No cultivar variation in shoot DW, N uptake and shoot N concentration could be proven at high N supply.

S uptake was also clearly decreased by low N, especially for cv Alaska and Expert, which had a significantly lower shoot S uptake than the other two cultivars (Table1). At high N supply cultivars did not vary in shoot S uptake. Nitrogen supply had no general effect on shoot S and sulfate-S concentration, however, the cultivar variation in shoot S and sulfate concentrations was affected by N supply. Cv Alaska had the lowest shoot S and sulfate-S concentrations and cv Expert had the highest sulfate-S concentration at low N supply. Shoot N:S ratio was decreased by low N supply. Cv Alaska had the highest shoot N:S ratio and cv Savannah the lowest ratio at low N supply, but no cultivar variation could be proven at high N supply. Sulfate-S:S ratio was increased at low N, but just for cv Expert, which had a higher ratio at low N supply than cv Alaska and Savannah.

3.2 Leaf parameters

Generally, high N supply significantly increased leaf dry weight, N concentration and N:S ratio, while no obvious N effects were found on leaf S concentration, sulfate-S concentration and sulfate-S:S ratio (Table 2, 3). The senescence process induced by covering lead to a decrease in leaf dry weight and leaf N concentration at low N supply. At high N supply, the dry weight loss was less severe, which resulted in higher N concentrations in senescent leaves compared to mature leaves. Leaf sulfur concentration was higher in senescent leaves than mature leaves both at low and high N supply. Due to a higher remobilization of nitrogen compared to sulfur at low N supply, N:S ratio decreased in senescent leaves, while at high N supply, the N:S ratio remained unchanged during the senescence progress. The sulfate-S-concentration at low N supply was more increased in senescent leaves than total S-concentration, so that the sulfate-S:S ratio slightly increased in senescent compared to mature leaves. At high N supply, however, sulfate-S:S ratio remained roughly unchanged between mature and senescent leaves.

Besides N and covering effects described above, significant cultivar effects were found for most leaf DW, N, and S parameters, except for sulfate-S:S ratio (Table 2, 3).

In mature leaves, cvs Savannah and Alaska accumulated an around 1 fold higher leaf DW than cv Expert, which also led to a significantly lower leaf N concentration in these two cultivars compared to cv Expert at low N supply. At high N supply, however, cultivars did not differ in leaf DW and N concentration. Clear cultivar differences were also found in S and sulfate-S concentrations of mature leaves, especially at low N supply, where cv Alaska had the lowest S and sulfate-S concentrations and the highest N:S ratio. A similar trend could be observed at high N supply. No cultivar variation was found in sulfate-S:S ratio in mature leaves at both low and high N supply.

In senescent leaves, the cultivars did not differ in leaf DW at both low and high N supply. Nevertheless, cv Savannah had a significantly lower leaf N concentration than cv Expert at low N supply, but contained the highest N concentration of all cultivars at high N supply. This cultivar also exhibited the highest S concentration in senescent leaves at low N, and cv Alaska the lowest. At high N supply, however, cvs Savannah and Aragon showed significantly higher S concentrations in senescent leaves than cvs Alaska and Expert. Similar cultivar differences as for total S concentrations were also found for the sulfate-S concentrations at both low and high N supply, resulting in similar sulfate-S:S ratios for all cultivars.

3.3 Apparent remobilization efficiencies

Leaf biomass, N and S apparent remobilization efficiencies were clearly affected by N supply (Fig. 1). On average, cultivars remobilized 67% of the biomass present in mature leaves prior to leaf death, 73% of the nitrogen, and 39% of the sulfur at low N supply, while at high N supply, the values amounted to 34, 21, and 16% for biomass, N, and S, respectively. Thus, the mean leaf biomass, N and S remobilization efficiencies were 2.0, 3.5 and 2.5 times higher at low N supply than at high N supply, respectively.

Moreover, cultivars also differed significantly in leaf biomass, N and S remobilization efficiency, with the cultivar differences mainly existing at low N supply (Fig. 1). At low N supply, cv Savannah had the highest biomass remobilization, and cv Expert the lowest, while cvs Aragon and Alaska ranged in the middle. The same cultivar differences could be found for the nitrogen remobilization, and to a slightly lower degree also for the sulfur remobilization.

At high N supply, no cultivar variation in leaf biomass, N and S remobilization could be proven. For leaf biomass remobilization efficiency, this resulted in a significant N × cultivar interaction, while an interaction could not be proven for N and S remobilization efficiency.

4 Discussion

The underlying hypothesis of the study was that under N-limiting conditions sulfur is preferentially stored in the form of sulfate in mature leaves, which causes a restriction of subsequent sulfur remobilization and may thus cause physiological sulfur deficiency in growing plant organs. The results of the study demonstrated that the assumption of a higher sulfate accumulation under N-limiting conditions did not prove true. Sulfate-S concentrations were not increased under low N supply, neither in absolute amounts, nor relative to total S concentrations (Table 2). Obviously, the putative decrease in OAS concentrations due to N limitation (Kim *et al*., 1999) was not the dominating factor in the regulation of sulfur assimilation. Instead, other regulating factors, like concomitant low abundance of reduced sulfur compounds, which can down-regulate sulfur assimilation (Hawkesford, 2000), must have been more decisive.

A similar conclusion can be drawn for the sulfur uptake (Table 1). Although sulfur uptake was clearly reduced under N limitation, this reduction was not as strong as for N uptake. Sulfur uptake thus seems to be regulated by factors related to overall plant growth rather than by the N nutritional status, resulting in similar S concentrations in the dry matter at low and high N supply (Table 1, 2).

Total S remobilization efficiency was even higher at low compared to high N supply (Fig. 1). An increased sulfur remobilization under combined N and S limitation has already been found for soybean (Sunarpi and Anderson, 1997a, b) and was caused by proteolysis of otherwise insoluble S forms induced by N starvation. The degree of S remobilization found in the current study (Table 1, Fig. 1) suggests that at low N supply most of the remobilized sulfur was derived from insoluble S forms and only a minor part from sulfate-S.

Summarizing, although most of the sulfur taken up was stored in the form of sulfate, which was poorly remobilized, the degree of S uptake and remobilization at low N supply was not

specifically reduced due to N limitation and may thus still be as sufficient as at high N supply for the S demand of growing plant organs.

The winter oilseed rape cultivars used in this study showed a considerable variation in S uptake (Table 1) and in biomass, N and S remobilization efficiencies (Fig. 1). This corroborates previous investigations of genotypic variation in S efficiency of canola varieties (Balint and Rengel, 2011). In their study, the genotypes also varied in S uptake and remobilization like in the present study, while S assimilation was not investigated.

The reasons for the genotypic variation in S uptake cannot be resolved in detail by the results of the present study, but it can be stated that it was not solely related to genotypic variation in growth or N uptake, particularly the low S uptake of cv Alaska could not be related to these two factors.

The genotypic variation in S remobilization efficiency, in contrast, was closely related to biomass and N remobilization (Fig. 1). It seems that both the N and S remobilization require an accumulation of dry matter in the mature leaf, as can be seen for cv Savannah, to provide sufficient C assimilates for the transport of N and S. An accumulation of sugars in mature leaves which will be degraded to organic acids during senescence (Masclaux et al., 2001) might therefore be important for assimilate retranslocation. At high N supply, however, C assimilates will be more preferentially consumed for N assimilation and subsequent protein formation, and will later be not available for assimilate retranslocation.

In conclusion, although it might not be expected that N deficiency can cause physiological S deficiency, there was a substantial genotypic in S metabolism that is expressed under low N supply only, and might become growth-limiting for individual cultivars under stress conditions. If this is related only to low-N stress, or also to other stress conditions, needs further investigation.

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Figure and table legends

Figure 1: Apparent remobilization efficiency in (A) leaf biomass, (B) nitrogen and (C) sulfur of four winter rape cultivars grown at two N rates. Same letter means not significantly different at P <0.05 level within each N rate (Tukey test). ***, P < 0.001; **, P < 0.01; ns, not significant at P <0.05 level.

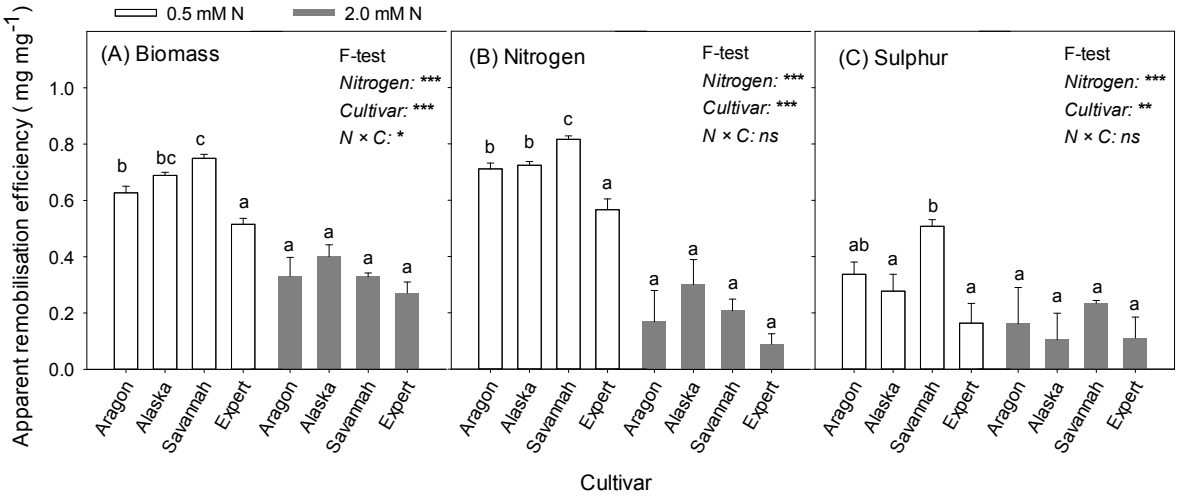
Table 1: Shoot dry weight, N and S parameters of four winter oilseed rape cultivars grown at two N rates (0.5 and 2.0 mM). Cultivar means with the same letter are not statistically different at P < 0.05 level within each N rate. ***, P < 0.001; **, P < 0.01; *, P < 0.05; ns, not significant at P < 0.05 level.

Table 2: Dry weight, N and S parameters of senescent and fully expanded leaf 3 of four winter oilseed cultivars grown at two N rates (0.5 and 2.0 mM). Cultivar means with the same letter are not statistically different at P < 0.05 level within each N rate.

Table 3: Analysis of variance showing the significance probability (Pr > F) of the N rate (N), cultivar (Cult.), covering (Cov.) and their interaction effects on leaf 3 dry weight, N and S parameters of four winter oilseed cultivars grown at two N rates (0.5 and 2.0 mM). ***, P < 0.001; **, P < 0.01; *, P < 0.05; +, P < 0.1; ns, not significant.

Figure

Figure 1



Tables

Table 1

N rate (mM)	Cultivar	DW (g plant^{-1})	N uptake (mg plant^{-1})	S uptake (mg plant^{-1})	N concentration (mg g^{-1})	S concentration (mg g^{-1})	N: S ratio (mg mg^{-1})	Sulfate-S Concentration (mg g^{-1})	Sulphate-S: S ratio (mg mg^{-1})
0.5	Aragon	18.8 b	263.4 b	157.9 b	14.1 a	8.5 ab	1.67 ab	5.71 ab	0.67 ab
	Alaska	17.0 b	259.4 b	113.9 a	15.3 a	6.7 a	2.28 c	3.76 a	0.56 a
	Savannah	15.7 b	233.8 b	161.6 b	14.9 a	10.3 b	1.45 a	6.42 bc	0.62 a
	Expert	10.9 a	196.4 a	104.2 a	18.0 b	9.6 b	1.88 b	7.46 c	0.77 b
	Mean	15.8	239.7	138.0	15.4	8.9	1.78	5.87	0.65
2.0	Aragon	34.1 a	781.1 a	257.7 a	22.9 a	7.5 a	3.07 a	4.80 a	0.64 a
	Alaska	27.3 a	717.7 a	243.1 a	26.4 a	9.0 a	2.96 a	5.18 a	0.58 a
	Savannah	31.5 a	744.7 a	287.5 a	24.7 a	9.5 a	2.59 a	5.60 a	0.57 a
	Expert	23.4 a	676.0 a	218.4 a	28.8 a	9.3 a	3.09 a	5.43 a	0.58 a
	Mean	27.8	718.8	244.7	26.4	8.9	2.96	5.27	0.59
	N	***	***	***	***	ns	***	ns	*
	Cult.	***	*	***	**	**	***	*	*
	N × Cult.	ns	ns	ns	ns	*	*	*	*

Table 2

		Senescent					Fully expanded				
	N rate (mM)	Aragon	Alaska	Savannah	Expert	Mean	Aragon	Alaska	Savannah	Expert	Mean
0.5	DW (g)	0.25 a	0.29 a	0.28 a	0.24 a	0.26	0.66 ab	0.93 bc	1.10 c	0.49 a	0.80
	N concentration (mg g^{-1})	9.3 ab	9.3 ab	7.8 a	13.0 b	9.9	12.8 ab	10.5 a	10.5 a	14.6 b	12.1
	S concentration (mg g^{-1})	21.6 b	12.5 a	24.5 c	19.0 b	19.4	12.8 b	5.5 a	12.6 b	11.2 b	10.4
	N: S ratio (mg mg^{-1})	0.43 a	0.76 b	0.32 a	0.71 b	0.55	1.00 a	2.07 b	0.84 a	1.31 a	1.32
	Sulphate-S concentration (mg g^{-1})	20.6 ab	13.1 a	22.5 b	17.0 ab	18.3	10.8 b	4.5 a	9.9 b	9.4 b	8.3
	Sulphate-S: S ratio (mg mg^{-1})	0.95 a	0.93 a	0.92 a	0.89 a	0.92	0.85 a	0.78 a	0.72 a	0.85 a	0.80
2.0	DW (g)	0.50 a	0.46 a	0.50 a	0.50 a	0.49	0.75 a	0.77 a	0.75 a	0.68 a	0.72
	N concentration (mg g^{-1})	28.0 a	35.8 ab	40.1 b	33.9 ab	34.9	22.6 a	32.2 a	34.0 a	26.5 a	27.7
	S concentration (mg g-1)	18.6 b	15.2 a	19.0 b	14.2 a	16.4	14.9 bc	9.3 a	16.7 c	11.8 ab	12.6
	N: S ratio (mg mg^{-1})	1.50 a	2.34 b	2.11 b	2.42 b	2.17	1.51 a	3.08 b	2.04 ab	2.30 ab	2.25
	Sulphate-S concentration (mg g^{-1})	18.1 b	12.7 a	15.1 ab	11.8 a	13.9	13.4 ab	8.8 a	15.4 b	9.0 a	11.1
	Sulphate-S: S ratio (mg mg^{-1})	0.93 a	0.83 a	0.79 a	0.83 a	0.85	0.90 a	0.92 a	0.92 a	0.76 a	0.87

Table 3

Factor	Dry weight	N concentration	S concentration	N:S ratio	Sulphate-S concentration	Sulphate-S:S ratio
N	*	***	ns	***	ns	ns
Cult.	**	**	***	***	***	ns
Cov.	***	*	***	+	***	+
N × Cult.	*	***	***	*	+	ns
N × Cov.	***	***	***	***	***	*
Cult. × Cov.	**	ns	ns	*	ns	ns
N × Cult. × Cov.	ns	ns	ns	ns	ns	ns

Chapter 3

Genotypic Variation in Leaf Sulfur Metabolites in Oilseed Rape in Relation to Nitrogen Remobilization

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Genotypic Variation in Leaf Sulfur Metabolites in Oilseed Rape in Relation to Nitrogen Remobilization

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7 figures

Running title: Genotypic Variation in Leaf Sulfur Metabolites in Rapeseed

Key words: N remobilization; Sulfur metabolites; Genotypic variation; Glucosinolates; *Brassica napus* L.

Abstract

Aims Winter oilseed rape (*Brassica napus* L.) is often characterized by low N harvest indices. It is hypothesized that an improvement of reproductive growth by a high sulfur supply to developing organs could improve reproductive growth and thus N harvest index. In this study it is tested, if genotypic variation in S distribution to developing organs exists.

Methods Genotypic variation in S fractions in sulfate, glucosinolate (GSL), glutathione (GSH) and the insoluble-S within oilseed rape leaves were investigated for 13 double low (DL) and 10 conventional high GSL (HG) cultivars at low N supply. S distribution to developing organs as well as N remobilization from mature leaves was also examined.

Results DL cultivars were found to have less S distributed to GSL (6.4% vs 12.8%) and the insoluble fraction (3.7% vs 13.0%), but significantly more in sulfate (89.9% vs 74.2%) in mature leaves than the HG cultivars, resulting in a low ability in distributing S to developing leaves. In addition, DL cultivars showed a superior leaf N remobilization.

Conclusions The high S assimilation and distribution to developing leaves of high GSL cultivars seems to cause an inferior leaf N remobilization for these cultivars. It is speculated that there is a competition between nitrogen and sulfur for amino acid synthesis, causing the differences in N and S transport.

1 Introduction

Despite its high capacity to take up nitrate from the soil, winter oilseed rape (*Brassica napus* L.) is characterized by a very low N recovery in the reproductive tissues under field conditions (*Malagoli* et al. 2005). This is because a significant proportion of the N taken up was released to the soil in leaf litter before pod filling (*Malagoli* et al*.,* 2005; *Gombert* et al*.,* 2010), suggesting that the vegetative parts were not effective in mobilizing N to the pods during the reproductive period. However, it was shown that N remobilization from vegetative plant parts was most probably sink-limited at high N supply (*Gombert* et al., 2010), i.e. the growth of reproductive organs must be enhanced to improve N remobilization and thus N harvest index (NHI) and N recovery. In addition, it has been shown that NHI can be very high in oilseed rape and leaf N remobilization can be very efficient, suggesting that a poor N remobilization must rather be caused by sink limitation (*Ulas* et al*.,* 2013). Also at low N supply, production and growth of reproductive organs were found to be of decisive importance to achieve a high yield and N efficiency (*Balint* and *Rengel,* 2008). Due to economic and environmental reasons, the challenge in oilseed production lies in maintaining yield while using the minimum possible level of N fertilization (*Rathke* et al., 2006). This challenge could be met partly with the development of N-efficient genotypes with better reproductive growth.

It has been reported that sulfur mainly enhances the reproductive growth, more than the vegetative growth, and the proportion of the reproductive tissues (inflorescences and pods) in total dry matter was found significantly increased by sulfur during pod development (*McGrath* and *Zhao,* 1996). In contrast, sulfur deficiency suppressed the development of reproductive organs of rape and even led to pod abortion (*McGrath* and *Zhao,* 1996), which may further decrease N remobilization as a consequence of sink limitation.

Oilseed rape has a high demand for S because S is utilized not only for protein but also for glucosinolate (GSL) synthesis (*Holmes*, 1980). Conventional single low cultivars have been replaced by double low cultivars with the aim of increasing the proportion of rapeseed meal used in both ruminant and non-ruminant feeds (*Zhao* et al., 1993). Although it might be expected that double low cultivars have a lower S demand, it has been observed that double low cultivars were more susceptible to S deficiency than conventional cultivars (*Schnug* and *Haneklaus*, 1988; *Schnug,* 1989). In a field experiment at S deficient sites in Scotland, *Booth* et al. (1991) also found that yield responses of double low varieties to S application were much greater than those of a single low variety. The physiological and biochemical background for the sensitive response to low S has not yet been unequivocally elucidated. It has been suggested that GSL act as a store of S that can be recycled within the oilseed rape plant to support growth in S deficient situations and that double low cultivars were more sensitive to S deficiency because of their low GSL content (*Schnug*, 1991). However, GSL was found unlikely to be a major store of recyclable S in oilseed rape crops, because GSL-S accounted for less than 8% of the total S (*Fieldsend* and *Milford,* 1994). Another explanation for the high S demand in oilseed rape has been the high sulfate accumulation in old leaves, which is not further used for plant growth (*Hawkesford,* 2000). Although sulfate can be remobilized from the vacuoles and be translocated by the phloem stream, the remobilization of sulfate is considered to be very slow (*Bell* et al., 1995a, b). This was further supported by a more detailed investigation about sulfur distribution within oilseed rape leaves, where it was clearly shown that neither GSL nor glutathione (GSH) were major sources of S during S deficiency. On the contrary, sulfate was supposed to be the most important S source (*Blake-Kalff* et al., 1998). Beside the difference in GSL content, it seems thus likely that double low cultivars and high GSL cultivars may also differ in sulfate accumulation, which might explain the different performance in S distributing into developing parts.

To our knowledge, however, a comprehensive study of genotypic variation in S metabolites such as GSL, GSH and sulfate, is so far not available. Moreover, how the genotypic variation in S metabolites contribute to the difference in S supply of growing organs is yet unknown. Furthermore, it has been clearly demonstrated that S assimilation, GSL biosynthesis and N assimilation are interdependent (*Halkier* and *Gershenzon,* 2006; *Huseby* et al., 2013; *Yatusevich* et al., 2010). Hence, genotypic variation in S metabolites is likely to exert an influence on N assimilation and amino acid composition, which may further impact N remobilization. While the co-regulation of these processes is well established, the effects of genotypic variation in S metabolites on N remobilization were hardly investigated.

Therefore, we conducted a comparative study of genotypic variation in S metabolites with 23 winter oilseed rape cultivars, including 13 double low (DL) and 10 conventional high GSL (HG) oilseed rape cultivars, selected from a broad genetic background. Here we want to clarify if there is a considerable genotypic variation in the S distribution to growing organs which can already be assessed in short-term experiments. We hypothesize that genotypic variation in sulfate accumulation is the main contributor to differences in S distribution in developing leaves. The results will allow us to conclude whether genotypes with improved S distribution patterns can be selected, which might be related to improved reproductive growth under field conditions.

2 Materials and methods

2.1 Hydroponic experiments

Hydroponic experiments were performed with 13 DL and 10 HG winter oilseed rape cultivars. The 23 rapeseed cultivars were selected from over 1000 cultivars from the PreBreedYield (PBY) breeding program in order to present a broad genetic background. The 23 cultivars used in this study were also chosen because of their adaptation to local growing conditions.

The seeds were supplied by Norddeutsche Pflanzenzucht Lembke (NPZ, Hohenlieth, Germany). The experiments were repeated four times from October 2012 till June 2013 in the greenhouse of the Institute of Plant Nutrition and Soil Science, Kiel University, Germany. Each individual experiment lasted 62 to 66 days.

Seeds were germinated in the greenhouse (day/night temperature 25/18℃.) using a "sandwich" method: the seeds were arranged between filter paper sandwiched between sponges and PVC-plates on both sides. The "sandwiches" were fixed by rubber bands and kept in a box containing tap water. After germination, four seedlings from each cultivar were transplanted into continuously aerated nutrient solution in 10 L plastic pots and thinned to two seedlings per pot after 8 days. Nutrient solution was supplied firstly at 25% strength and then increased to 50% strength on day 3. From day 5 on, plants received full nutrient solution with the following composition: 1000 µM KCl, 500 µM $CaCl_2$, 200 µM K_2HPO_4, 75 µM KH_2PO_4, 300 µM $MgSO_4$, 10 µM H_3BO_3, 2 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.2 µM $CuSO_4$, 0.05 µM $(NH_4)_2Mo_7O_{24}$ and 60 µM Fe-EDTA. $Ca(NO_3)_2$ and NH_4NO_3 were used as nitrogen source in a ratio of 90% nitrate and 10% ammonium. N concentration was set as 0.5 mM N from the beginning of the experiment until the third leaf (leaf 3) counted from the base of the plants appeared and then as 0.1 mM N until leaf 3 was fully expanded (18-22 days after transplanting). After that, 0.05 mM N was applied until the end of the experiment. Additional $CaCl_2$ solution was used to complement Ca to the set concentration (1 mM). The nutrient solution was replaced three times a week to maintain the set concentrations. Harvests were done individually for each cultivar when leaf 3 was shed from both plants in the pot. After harvest, plants were separated into leaf 3, mature leaves, developing leaves and the rest shoot, freeze-dried to constant weight and grounded into fine powder for further measurements.

2.2 Chemical analysis

Total N and S concentrations of each fraction were determined according to the Dumas combustion method using a CNS elemental analyzer (Flash EA 1112 NCS, Thermo Fisher Scientific, Waltham, MA, USA).

Sulfate concentration was determined by ion-chromatography (ICS 2500, Dionex, Sunnyvale, CA, USA). Sulfate extraction was done according the method as described by (*Gerendas* and *Sattelmacher,* 1997). 1.5 ml de-ionized water was added to 0.03 g of dried plant material and promptly transferred to a boiling water bath for 5 min. After incubation on ice for at least half an hour, samples were centrifuged and proteins were further excluded from supernatant by extraction with chloroform. Supernatant was filtered on C18-columns (Strata spe. 8B-S001-DAK) prior to ion-chromatography.

Total glutathione (glutathione (GSH) plus glutathione disulfide (GSSG)) in mature leaves was measured according to *Griffith* (1980) using an enzymatic method with GSSG solution as a standard. For GSH extraction, 150 mg ground sample material was added into 2 ml 1% BSA (w/v) solution. After vortex and centrifugation (12000 rpm, 5 min), 1 ml supernatant was transferred to 1ml sulfosalicylic acid (10%, w/v) and incubated on ice (3 min) to precipitate protein. After that, 540 µl supernatant was put to a new reaction tube with 60 µl triethanolamine (50%, v/v) and kept on ice until measurement. GSSG is reduced to GSH by the enzyme glutathione reductase (GR type III, SIGMA, 10 unit ml^{-1}) under NADPH + H^+ consumption (25%, w/v, containing 6.3 mM Na-EDTA). The reaction started with 100 µl 6 mM DTNB solution, and the absorption was measured with photometer every 20 s at 412 nm for 2.5 min.

Glucosinolates in mature leaves and leaf 3 were identified and quantified according to the method of Krumbein et al. (2005) with slight modifications. 0.2 g freeze-dried powder were extracted with 2 ml of 70% boiling methanol (10 min, 75°C water bath) with 100 µl of 5 mM

sinigrin solution (Sigma-Aldrich Co., MO, USA) as internal standard. Afterwards, 0.5 ml barium acetate (0.4 M) was added to precipitate proteins. After centrifugation (4000 rpm, 10 min, 25°C), the pellets were re-extracted twice. Supernatants were combined and made up to 5 ml with 70% methanol. 4.5 ml extract was loaded onto a 1 ml mini-column (JT Baker, USA) containing 200 µl of activated DEAE Sephadex™A25 (Amersham Biosciences, Sweden) for de-sulphate with aryl sulfatase (Sigma-Aldrich Co., MO, USA) over night. The resultant desulfo-GSL were eluted with 5 ml of double distilled water and stored at −20°C. Samples (20 µl) were analyzed in HPLC system (LC-10AT pump, CTO-10A column oven, SCL-10A VP system controller, Shimadzu, Kyoto, Japan) consisting of a UV–VIS detector (SPD-10A) set at 229 nm and a prontosil ODS2 column (250×4 µm, 5 µm, Bischoff, Germany). The mobile phase was ultra-pure water (A) and 20% acetonitrile (Tedia, USA) (B). The flow rate was 1.3 ml min^{-1}.

2.3 Calculations and statistics

N, S and sulfate-S content of each fraction was calculated as the product of concentration and DW. N remobilization efficiency (NRE) was estimated with the following equation:

$$NRE = \frac{[N]_{Mat.} - [N]_{Sen.}}{[N]_{Mat.}} \quad (1),$$

in which $[N]_{Mat.}$ and $[N]_{Sen.}$ are N concentration of the mature leaf and senescent leaf 3, respectively. S distribution into developing leaves ($SD_{Dev.}$) was evaluated by the ratio of S concentrations in developing and mature leaves:

$$SD_{Dev.} = \frac{[S]_{Dev.}}{[S]_{Mat.}} \quad (2),$$

where $[S]_{Dev.}$ and $[S]_{Mat.}$ are the concentrations in developing and mature leaves.

Relative percentage of insoluble S (mainly protein-S) was calculated by subtracting the percentages of sulfate-S, GSL-S and GSH-S from the total S content of the sample (= 100%).

Statistical tests were carried out with the software R 3.0.2. To test genotypic variation in S metabolites, ANOVA was conducted using general linear model with experiment and cultivar as factors. For the comparison between cultivar types an independent t-test was carried out using means of individual cultivars in four repeated experiments (DL, n = 13; HG, n = 10). Linear regressions were calculated to check the relationship between shoot sulfate:S ratio and $SD_{Dev.}$. Data were tested for normality prior to the statistical analysis. Differences between means were considered significant when the P-value of the ANOVA F-test was less than 0.05.

3 Results

3.1 Shoot dry matter, N and S uptake

The shoot dry weight (DW) and N uptake differed significantly between cultivars (Figure 1a, b). Shoot DW ranged between 5.9 g $plant^{-1}$ (PBY23) and 10.5 g $plant^{-1}$ (PBY13). Generally, cultivar ranking in N uptake showed a similar pattern as in shoot DW. Moreover, clear cultivar variation was also found in shoot S uptake (Figure 1c). Cv PBY61 showed the lowest shoot S uptake (34.8 mg $plant^{-1}$), while cv PBY02 was the best in shoot S uptake (62.3 mg $plant^{-1}$). Mean shoot N concentrations were 8.84 (ranging from 7.54 to 1.00 mg g^{-1}) and 8.87 mg g^{-1} (ranging from 7.67 to 1.00 mg g^{-1}) for DL and HG cultivars, respectively, while mean shoot S concentrations amounted to 5.38 (ranging from 4.23 to 7.12 mg g^{-1}) and 5.92 mg g^{-1} (ranging from 4.15 to 7.62 mg g^{-1}).

However, double low cultivars did not generally differ from high glucosinolate cultivars in shoot DW, N uptake or S uptake.

Significant experimental effects were found for shoot DW, N and S uptake (as well as for sulfate, GSH and GSL concentrations; Figures 2 and 4), indicating considerable variation between individual experiments caused by environmental effects even under greenhouse conditions.

3.2 Glucosinolate composition and concentration in mature leaves

Significant cultivar variation was observed in total GSL content in mature leaves (Figure 2a). Total GSL content in mature leaves of DL cultivars ranged from 1.20 to 5.92 μmol g^{-1} DW, while that of HG cultivars ranged from 4.41 to 13.04 μmol g^{-1} DW. On average, the HG cultivars had comparatively higher total GSL concentration (7.34 μmol g^{-1} DW) in mature leaves than the DL cultivars (3.80 μmol g^{-1} DW).

To clarify the major GSL family that caused cultivar variation in total GSL in mature leaves, we further analyzed cultivar performance in the concentration of each GSL family (Figure 2b, c and d). Firstly, the aliphatic family was the dominant GSL family with much higher concentrations than the other two GSL families. Moreover, significant cultivar differences were observed in aliphatic, indolic and aromatic GSL and a generally similar pattern in cultivar ranking was found in aliphatic GSL concentration as in total GSL concentration. On average, aliphatic and aromatic GSL concentrations detected in HG cultivars were more than 3-fold of that in DL cultivars (both $P < 0.001$). However, no HG versus DL type difference was observed in indolic GSL concentrations. Taken together, the aliphatic GSL family was the major contributor for cultivar variation in total GSL concentration in mature leaves.

In order to estimate the possible S remobilization from glucosinolates, the relative changes of individual GSL were evaluated by the ratio of the difference in GSL concentrations of mature and senescent leaves compared to the original mature leaf GSL concentration (Figure 3). Different individual GSL differed in the pattern of changes. Generally, 4-hydroxyglucobrassicin and glucobrassicin, belonging to the indolic family, decreased with

leaf senescence. However, the other three types of GSL from the indolic family accumulated in a number of cultivars, resulting in an accumulation of indolic GSL in senescent leaves. In contrast, an obvious reduction was observed in all 5 aliphatic GSL, except a slight increase of progoitrin in cv PBY24 and of glucoraphanin in cv PBY02 and cv PBY25. Moreover, gluconasturtiin, the only GSL from the aromatic family, also decreased strongly. It seems thus that most of the S remobilization from GSL must have come from aliphatic GSL, in view of both absolute concentrations in mature leaves and the relative changes.

3.3 Mature leaf S concentrations and binding forms of sulfur in the leaves

Beside cultivar difference in GSL concentration, pronounced cultivar differences were also proven in mature leaf total S, sulfate-S, and GSH-S concentrations (Figure 4). The total S concentration ranged from 87.4 (cv PBY15) to 183.9 μmol g^{-1} DW (cv PBY04). Generally cultivar ranking in mature leaf sulfate-S concentration was similar as that in total S concentration, because the major part of mature leaf S was present in the form of sulfate in this study. Cv PBY23 was the highest in GSH-S concentration, with which 0.37 μmol g^{-1} DW GSH-S was detected. In contrast, cv PBY17 had the lowest GSH-S concentration (0.12 μmol g^{-1} DW). Regarding the cultivar types, DL cultivars were significantly higher in sulfate-S concentration than the HG cultivars, but no cultivar type difference was found in total S or GSH-S concentration.

To further investigate the differences in S binding forms in the leaves, the relative percentage of S in each metabolite to total S was calculated (Figure 5). The average sulfate-S:S percentage in DL cultivars was 89.9%, and thus significantly higher than that of HG cultivars (74.2%). Obviously, sulfate-S was the main form of S in both DL and HG cultivars. In contrast, the average GSL-S:S and insoluble-S:S percentage in DL cultivars was only 6.4% and 3.7%, significantly lower than that in HG cultivars (12.8% and 13.0%, respectively).

However, DL and HG cultivars did not differ in average GSH-S: S percentage (both around 0.14%).

3.4 S distribution to developing leaves and N remobilization from mature leaves

S distribution to developing leaves was evaluated by the ratio of S concentrations in developing and mature leaves ($SD_{Dev.}$, Figure 7a). Cultivars differed significantly in $SD_{Dev.}$. Cv PBY14 had the highest $SD_{Dev.}$, while cv PBY18 was the lowest. HG cultivars had a generally higher $SD_{Dev.}$ compared to DL cultivars. The mean $SD_{Dev.}$ of HG cultivars was 1.87 (ranging from 1.60 to 2.29), significantly higher than that of DL cultivars (mean 1.45, ranging from 1.00 to 1.82). As sulfate was the major form of sulfur in the present study, it is likely that the cultivar difference in sulfate-S may play a key role in the variation of S distribution into developing parts. To further confirm this, linear regression analysis was performed to check the relation between shoot sulfate-S:S ratio and S distribution into developing leaves ($SD_{Dev.}$). As expected, a close, negative relation was found with DL cultivars ($R^2 = 0.44$, $P < 0.001$), but it was not the case with HG cultivars (Figure 6).

In addition, significant cultivar variation was also observed in mature leaf N remobilization (Figure 7b). The highest NRE (0.47) was observed with cv PBY23, indicating the best N remobilization in mature leaves prior to leaf death. Cv PBY01 had the lowest NRE, with only 0.29. The average NRE of DL cultivars was 0.40, significantly higher than that of HG cultivars (0.36).

4 Discussion

4.1 High glucosinolate cultivars are superior in S distribution to developing leaves

The study is the first to quantify the genotypic variations in various S metabolites in mature leaves as well as their potential importance for S distributing into developing issues, using a range of DL and HG cultivars.

DL cultivars showed a significantly lower ability in distributing S into developing leaves than HG cultivars (Figure 7a). This may explain that DL cultivars seem to be more susceptible to low S, as reported previously (*Schnug* and *Haneklaus*, 1988; *Schnug*, 1989; *Booth* et al., 1991). No differences were found in either shoot S uptake (Figure 1c), or mature leaf S concentration (Figure 4a), which supports previous observations (*Schnug* and *Haneklaus*, 1988). Shoot S uptake or mature leaf S accumulation were thus not the reasons for cultivar differences in S distribution into developing leaves.

DL cultivars contained less glucosinolate in mature leaves than the HG cultivars (Figure 2d), resulting in a significantly lower GSL-S to total S percentage (Figure 5). This was in accordance with previous studies (*Fieldsend* and *Milford,* 1994; *Schnug* and *Haneklaus*, 1988). Previously, it has been reported that GSL in vegetative tissues at GS 6.3 accounted for 8% and less than 5% of total S in single low and DL cultivars (*Fieldsend* and *Milford*, 1994). However, the relative percentage of mature leaf GSL-S to total S in the present study was 12.8% for the HG cultivars, and 6.4% for the double low cultivars (Figure 5), which was slightly higher than that found previously, indicating that the relative contribution of GSL-S to total S may differ with different cultivars and growing conditions. In addition to the lower GSL-S to total S percentage, DL cultivars also had a significantly lower insoluble-S to total S percentage than the HG cultivars. This supports the recently found observation that GSL biosynthesis is highly co-regulated with sulfate assimilation (*Huseby* et al., 2013). In addition, we further revealed a significantly higher mature leaf sulfate-S accumulation in DL cultivars (Figure 4b), resulting in an also significantly higher sulfate-S to total S percentage. However, the DL and HG cultivars did not differ in mature leaf GSH-S (Figure 4c). The GSH-S contributed less than 0.2% of total S in this study, which was in accordance with previous study where the contribution of GSH-S to total S was found negligible (*Blake-Kalff* et al., 1998).

The main contributing factor for differences in S distribution in developing leaves thus seems to have been the degree of sulfate accumulation. In the present study the average mature leaf sulfate-S to total S percentage was 89.9% and 74.2% for DL and HG cultivars, respectively. This corroborated previous investigations of S distribution in oilseed rape leaves, where 70-90% of total S was in the form of sulfate (*Blake-Kalff* et al., 1998). Taken together both the absolute concentration and the relative contribution to total sulfur, it was obvious that sulfate-S was the major S source in mature leaves. It has been reported that sulfate stored in the vacuoles of mesophyll cells was only released under conditions of prolonged S stress and that this release was too slow to support new growth (*Bell* et al. 1995a; *Clarkson* et al., 1983). It thus seems likely that the higher mature leaf sulfate accumulation in DL cultivars might be the main reason for the lower S distribution into developing leaves. To further confirm this assumption at whole shoot level, linear regression analysis revealed a close, negative relationship between shoot sulfate-S: S and $SD_{Dev.,}$ for the DL cultivars ($R^2 = 0.44$, $P < 0.001$), indicating that higher shoot (mainly mature leaves) sulfate accumulation was the main reason resulting in the lower S distribution into developing leaves and thus higher susceptibility to S deficiency in DL cultivars. This relationship did not hold true for the HG cultivars ($P = 0.28$), indicating that for this cultivar group there is more genotypic variation in other S compounds with importance for S remobilization. The observations in this study suggested that GSL were unlikely to be a major store of recyclable S in rape crops, in view of low concentration and percentage relative to total S. However, this does not preclude the possibility that GSL acted as a minor contributor to plant overall S turnover and balance, especially in HG cultivars.

Moreover, we further revealed that the aliphatic GSL were the dominant GSL family responsible for remobilization due to both the high absolute concentration and relative changes during leaf senescence. This corresponds with the previous results with rapeseed (*Blake-Kalff* et al., 1998). In their experiment, the GSL concentration increased in the youngest leaves of plus-S plants grown on low N, mainly attributable to a 15-fold increase in

the concentration of aliphatic glucosinolates during the experiment. Further, a quantitative survey of GSL variation in 39 *Arabidopsis* ecotypes showed a significant positive correlation between the levels of aliphatic GSL in leaves and seeds, suggesting that aliphatic GSL were transported from the leaves to the seeds (*Kliebenstein* et al., 2001). All the research emphasizes the importance of aliphatic GSL for remobilization.

4.2 High glucosinolate cultivars are inferior in mature leaf nitrogen remobilization

Although HG cultivars were superior in S distribution into developing organs, they were also characterized by lower N remobilization efficiency (Figure 7). Former studies with soybean have shown that S and N were both exported from mature leaves after proteolysis and that this process was promoted at low N but inhibited at high N supply (*Sunarpi* and *Anderson,* 1997a, b). However, the results of our study rather suggest a trade-off between N and S remobilization. This contradiction can probably be explained by the fact that S assimilates were extremely abundant in HG cultivars. In contrast, such a trade-off did not occur with a lower level of assimilated S as observed in DL cultivars in the present study.

Previous studies have demonstrated that the major transport form of reduced N were amino acids, which were transported predominantly in the phloem (*Riens* et al., 1991). Although the studies about the regulation of amino acids export has been challenging, there was evidence that amino acids are transported in the phloem predominantly as glutamine, asparagine, glutamate and aspartate and these four amino acids may play a key role in phloem N remobilization (*Masclaux-Daubresse* et al*.,* 2008; *McAllister* et al*.,* 2012; *Okumoto* and *Pilot*, 2011; *Xu* et al., 2012). In particular, glutamine was reported as the most important amino acid for phloem N transport in rapeseed (*Möllers* et al., 1996; *Balint* and *Rengel,* 2011). On the other hand, the S-containing amino acids methionine (in rapeseed) and methionine derivate S-methylmethionine (in Arabidopsis and wheat) were the main contributors to phloem S transport (*Balint* and *Rengel,* 2011; *Bourgis* et al., 1999; *Lee* et al., 2008). Glutamine has a

C:N ratio of 5:2, while methionine has a ratio of 5:1. It is thus likely that glutamine is more efficient for N transport. More S-containing amino acids relative total amino acids can therefore lead to a decrease in phloem N remobilization efficiency. In the present study, the higher sulfate accumulation in DL cultivars resulted in significantly less insoluble-S (mainly protein-S) relative to total S (Figure 5), suggesting that the percentage of S-containing amino acids relative to total amino acids were lower in DL cultivars than that in the HG cultivars. As expected, significantly higher N remobilization efficiency was observed in DL cultivars compared with HG cultivars. However, the relationship between N and S remobilization via amino acids might be much more complex than just reflecting a competition for C assimilates. Increased levels of S-methylmethionine in transgenic pea plants were found to increase N translocation to the seeds (*Tan* et al. 2010), which is in contrast to our results and interpretations. The authors speculated that this effect must have been due to specific signaling effects imposed by S-methylmethionine. In case of the HG cultivars in this study, another S-containing metabolite with specific effects on N metabolism might as well play a role.

In summary, our results suggested the low ability in distributing S into developing leaves of DL cultivars was caused mainly by a high mature leaf sulfate accumulation, which is poorly re-allocated. On the other hand, the lower S assimilation seems to have caused a better N remobilization in DL cultivars compared with HG cultivars. This work shed some light on cultivar differences in S metabolites and N remobilization during vegetative growth. Assuming the superior leaf N remobilization of DL cultivars still exists during reproductive growth, a higher N harvest index and thus higher yield might be expected. Results from field experiments addressing this expectation will be presented in a separate paper.

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Figure legends

Figure 1: Shoot dry weight (a), N uptake (b) and S uptake (c) of 23 rapeseed cultivars grown under low N supply in hydroponics for around 64 days. The results of F-test are also given (Exp.: experiment; Cult.: cultivar; ***, $P < 0.001$). The graphs inserted show the comparison (ns, not significant at $P < 0.05$ level, t-test) between means of double low (DL, n = 13) and high glucosinolate (HG, n = 10) cultivars. Error bars represent the standard error of cultivars in each group.

Figure 2: Total glucosinolate (GSL) (a), aliphatic (b), indolic (c) and aromatic GSL (d) concentration in mature leaves from 23 rapeseed cultivars grown under low N supply for around 64 days. The results of F-test are also given (Exp.: experiment; Cult.: cultivar; ***, $P < 0.001$; ns, not significant). The graphs inserted are comparison (***, $P < 0.001$; ns, not significant at $P < 0.05$ level, t-test) between means of double low (DL, n = 13) and high glucosinolate (HG, n = 10) cultivars. Error bars represent standard error of cultivars in each group.

Figure 3: Relative changes (%) of individual glucosinolate (GSL) concentration of senescent leaves compared to mature leaves for 23 rapeseed genotypes grown under low N supply for around 64 days. 4-OHGBS, 4-Hydroxyglucobrassicin; 4OMGBS, 4-Methoxyglucobrassicin; GBS, glucobrassicin; NGBS, neoglucobrassicin; GIB, glucoiberin; PRO, progoitrin; GRA, glucoraphanin; GNA, gluconapin; GBN, glucobrassicanapin; GST, gluconasturtiin; TGSL, total GSL.

Figure 4: Sulfur (a), sulfate-S (b), and GSH-S (c) concentrations in mature leaves from 23 rapeseed cultivars grown under low N supply for around 64 days. The results of F-test are also given (Exp.: experiment; Cult.: cultivar; ***, $P < 0.001$). The graphs inserted are comparison (***, $P < 0.001$; ns, not significant at $P < 0.05$ level, t-test) between means of double low (DL, n = 13) and high glucosinolate (HG, n = 10) cultivars. Error bars represent standard error of cultivars in each group.

Figure 5: Relative percentage of S in various forms (Insoluble S, S in gluthatione (GSH-S), S in glucosinolates (GSL-S) and sulfate-S) in total S in mature leaves from double low (DL) and conventional high GLS (HG) cultivars. Comparison between two types of cultivars was done with t-test (***, $P < 0.001$; ns, not significant at $P < 0.05$ level). The relative percentage of GSH-S in total S was similar in DL and HG cultivars (both 0.14%, $P > 0.05$, not obviously seen in the graph).

Figure 6: Linear regression between shoot sulfate-S:S ratio and S distribution into developing leaves ($SD_{Dev.}$) for double low (DL) and high glucosinolate (HG) rapeseed cultivars. Error bars represent standard errors of values for each cultivar measured in four experiments.

Figure 7: Sulfur distribution into developing leaves ($SD_{Dev.}$, a) and mature leaf nitrogen remobilization efficiency (NRE, b) for 23 rapeseed grown under low N supply for around 64 days. The results of F-test are also given (Exp.: experiment; Cult.: cultivar; ***, $P < 0.001$). The graphs inserted are comparison (***, $P < 0.001$; **, $P < 0.01$, t-test) between means of double low (DL, n = 13) and high glucosinolate (HG, n = 10) cultivars. Error bars represent standard error of cultivars in each group.

Figures

Figure 1

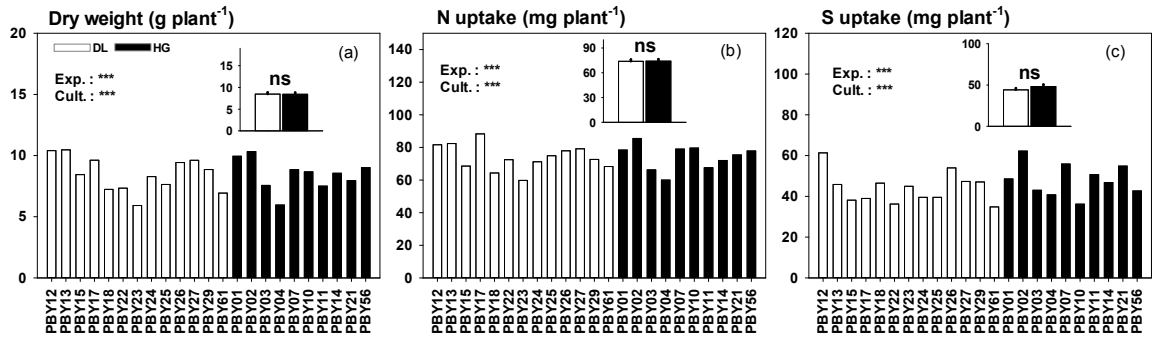


Figure 2

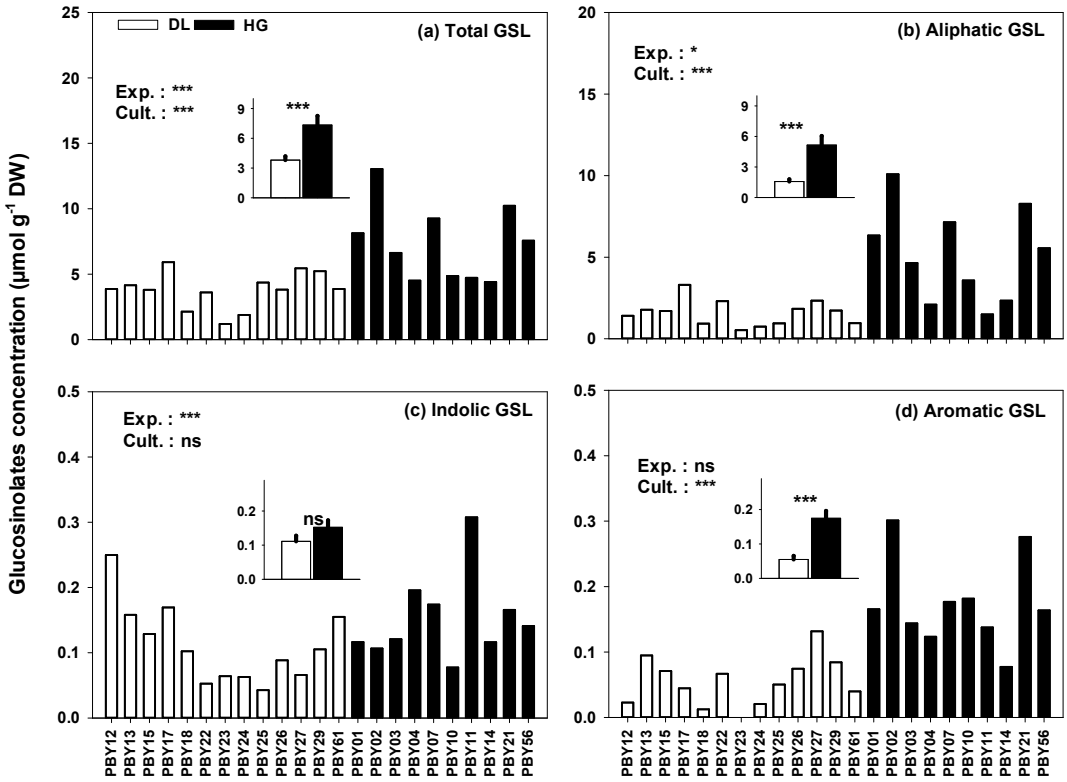

DL
HG
(a) Total GSL
Exp. : ***
Cult. : ***
(b) Aliphatic GSL
Exp. : *
Cult. : ***
(c) Indolic GSL
Exp. : ***
Cult. : ns
(d) Aromatic GSL
Exp. : ns
Cult. : ***
Glucosinolates concentration (µmol g-1 DW)
PBY12
PBY13
PBY15
PBY17
PBY18
PBY22
PBY23
PBY24
PBY25
PBY26
PBY27
PBY29
PBY61
PBY01
PBY02
PBY03
PBY04
PBY07
PBY10
PBY11
PBY14
PBY21
PBY56

Figure 3

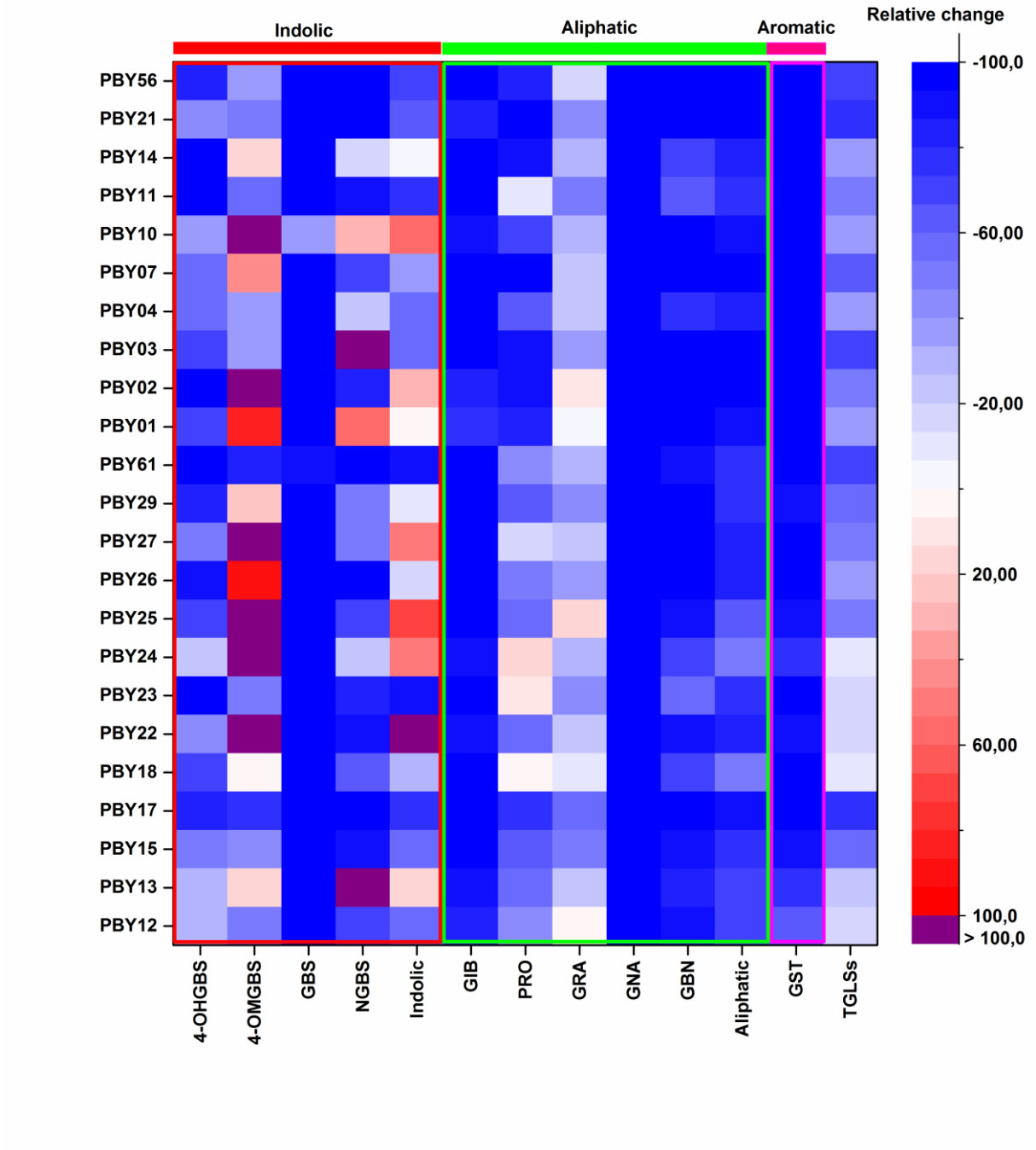

Indolic
Aliphatic
Aromatic
Relative change
-100,0
-60,00
-20,00
20,00
60,00
100,0
> 100,0
PBY56
PBY21
PBY14
PBY11
PBY10
PBY07
PBY04
PBY03
PBY02
PBY01
PBY61
PBY29
PBY27
PBY26
PBY25
PBY24
PBY23
PBY22
PBY18
PBY17
PBY15
PBY13
PBY12
4-OHGBS
4-OMGBS
GBS
NGBS
Indolic
GIB
PRO
GRA
GNA
GBN
Aliphatic
GST
TGLSs

Figure 4

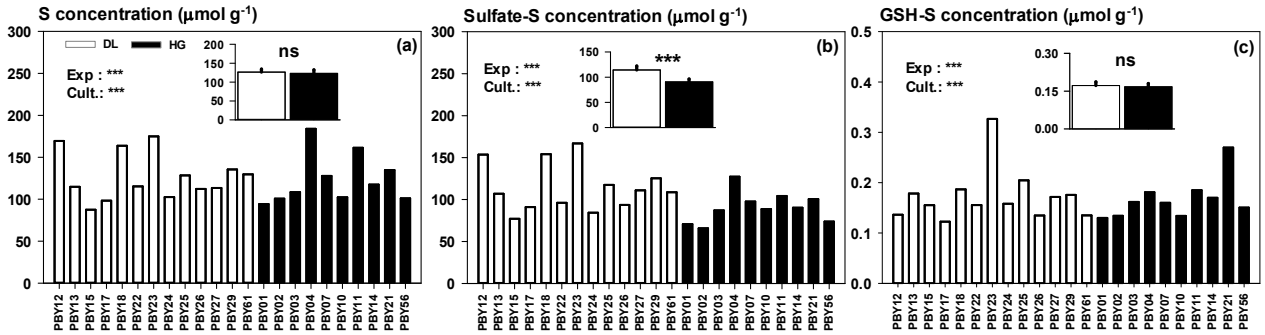

S concentration (µmol g-1)
DL
HG
Exp : ***
Cult.: ***
ns
(a)
Sulfate-S concentration (µmol g-1)
Exp : ***
Cult.: ***
***
(b)
GSH-S concentration (µmol g-1)
Exp : ***
Cult.: ***
ns
(c)
PBY12
PBY13
PBY15
PBY17
PBY18
PBY22
PBY23
PBY24
PBY25
PBY26
PBY27
PBY29
PBY61
PBY01
PBY02
PBY03
PBY04
PBY07
PBY10
PBY11
PBY14
PBY21
PBY56

Figure 5

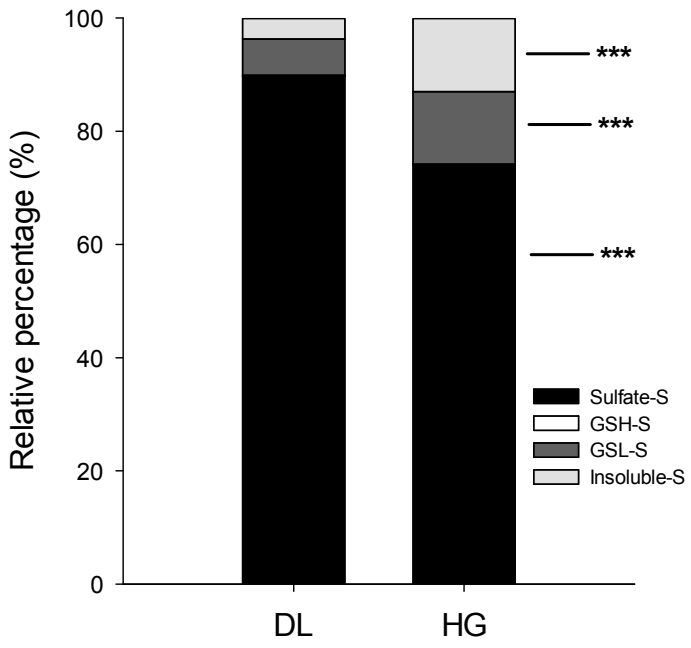

100
80
60
40
20
0
Relative percentage (%)
DL
HG
***
***
***
Sulfate-S
GSH-S
GSL-S
Insoluble-S

Figure 6

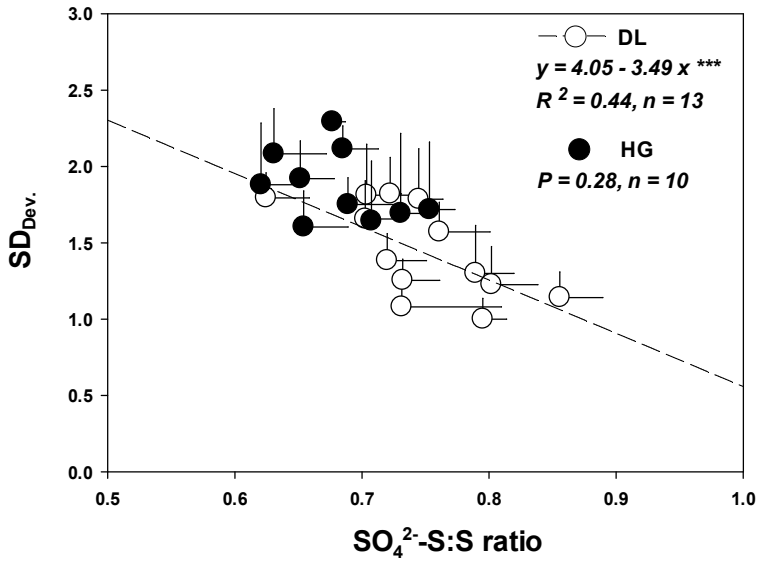
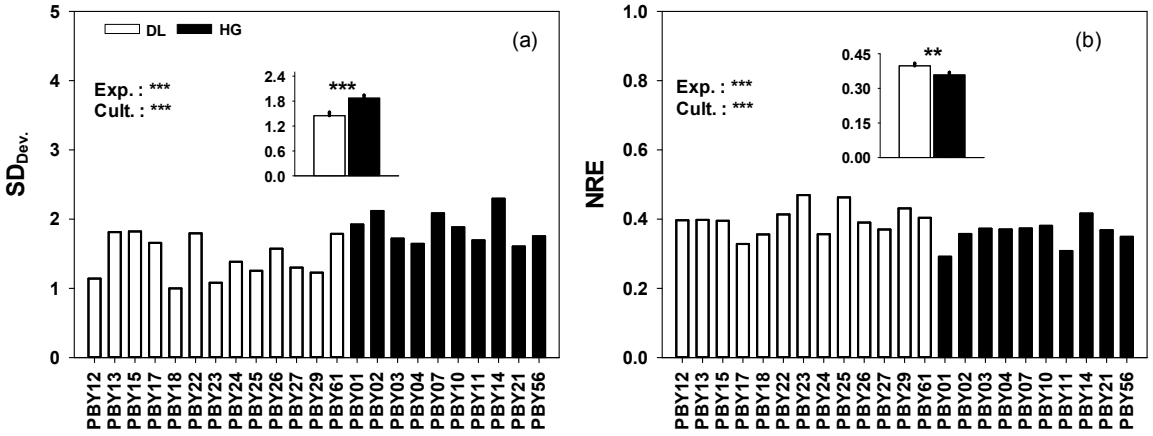

DL
y = 4.05 - 3.49 x ***
R 2 = 0.44, n = 13
HG
P = 0.28, n = 10
SDDev.
SO4 2- -S:S ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.5
0.6
0.7
0.8
0.9
1.0

Figure 7


DL
HG
(a)
Exp. : ***
Cult. : ***
***
SDDev.
(b)
**
NRE
PBY12
PBY13
PBY15
PBY17
PBY18
PBY22
PBY23
PBY24
PBY25
PBY26
PBY27
PBY29
PBY61
PBY01
PBY02
PBY03
PBY04
PBY07
PBY10
PBY11
PBY14
PBY21
PBY56

Chapter 4

Double low cultivars of oilseed rape (*Brassica napus* L.) perform better in nitrogen and sulfur efficiency than high glucosinolate cultivars

(In preparation)

Double low cultivars of oilseed rape (*Brassica napus* L.) perform better in nitrogen and sulfur efficiency than high glucosinolate cultivars

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5 figures

Key words: *Brassica napus* L.; N harvest index; genotypic variation; seed yield

Abstract

Sulfur (S) is particularly important for reproductive growth of rape, and sufficient S supply for reproductive growth might therefore be decisive for a high harvest index and subsequently also a high N efficiency of this crop. Since endogenous S is usually poorly remobilized, S supply to the pods might be limiting during reproductive growth. In the current study, a set of oilseed rape cultivars (cvs) differing in S distribution to developing organs, namely 13 double low (DL) and 9 high glucosinolate-containing (HG) cvs were grown in a three-location field experiment to identify the impacts of genotypic variation in S distribution on yield and N efficiency traits. It was hypothesized that a better S distribution into developing organs is related to higher harvest indices of the respective cultivars in the field. In accordance with the formerly observed higher S distribution into developing leaves during vegetative growth, HG cvs were 40% and 17% higher in S harvest index than DL cvs at low and high N rates. Also the mean seed S concentration of HG cvs were 2.58 and 2.39 fold of that observed with DL cvs at low and high N supply. However, the higher S distribution to seeds in HG cvs was not related to a high harvest index. Instead, DL cvs were significantly higher in harvest index and yield than the HG cv at both low and high N supply. Additionally, S distribution to developing leaves of HG cvs during vegetative growth was significantly and negatively correlated with N harvest index, and with yield at both low and high N conditions in the field experiments. Such relationships could not be proven for DL cvs. It was concluded that selection for favorable S distribution in rapeseed seedlings was feasible to reflect processes during reproductive growth in field experiments. However, selection of genotypes with improved capability to remobilize S to support reproductive growth was not decisive for improving yield capacity or N efficiency of oilseed rape.

Introduction

Winter oilseed rape (*Brassica napus* L.) is the most important oilseed crop in northern Europe. Rapeseed oil can be used for multiple purposes (edible oil, industrial purposes, biodiesel, etc.) and the cultivation of this crop is valuable for interrupting cereal-dominated crop-rotations. A characteristic of oilseed rape is its high sulfur (S) demand (Holmes 1980). Fertilizer recommendation rates for oilseed rape range between 20-100 kg S ha^{-1} compared to 10-50 kg S ha^{-1} for cereals (De Kok *et al.*, 2011). It has been reported that S is particularly important for reproductive growth of rape (Janzen and Bettany, 1984; McGrath and Zhao, 1996). A high S nutrition promotes the formation of yield components. Especially pod abortion is prevented with sufficient S supply (Zhao *et al.*, 1993; Fismes *et al.*, 2000), and also the number of seeds per pod and thousand kernel weight can be improved by enhanced S supply (Amanullah *et al.*, 2011). A sufficient S nutrition is therefore essential for achieving high harvest indices.

A high harvest index is thereby not only important for high yields, but it is also crucial for the nitrogen (N) economy of the crop. Nitrogen harvest indices have often been found to be low in oilseed rape, which contributes to the high N balance surplus of this crop (Aufhammer *et al.*, 1994). N remobilization from vegetative parts should therefore be improved, but has been found to be rather sink-limited at high N supply (Gombert *et al.*, 2010). An improved harvest index could therefore also contribute to improved N remobilization. Also at low N supply, harvest index is positively correlated to yield of oilseed rape cvs (Schulte auf'm Erley *et al.*, 2011; Nyikako *et al.*, 2014) and might therefore be an important contributor for a high genotypic N efficiency (i.e. yield at limiting N supply; Sattelmacher *et al.*, 1994). As a consequence, sufficient S supply for reproductive growth might be a decisive factor for the N efficiency of this crop.

There has recently been considerable effort to better characterize the S remobilization in oilseed rape (Dubousset *et al.*, 2009, 2010; Abdallah *et al.*, 2010, 2011). The S harvest index (SHI, i.e. the S amount in seeds divided by the total S in the shoot) has been found to be

noticeably lower than the N harvest index (NHI), indicating that S is remobilized to seeds less efficiently than N (Dubousset *et al.*, 2010). Leaves have been shown to be the primary donors of S for mobilization to seeds, which can supply up to 75% of mobilized S during reproductive development (Dubousset *et al.*, 2010; Gironde *et al.*, 2014). The stem may act also as a transient storage organ for remobilized S (Gironde *et al.*, 2014), while roots do not significantly contribute to endogenous S remobilization (Dubousset *et al.*, 2010). As the majority of S accumulated in leaves is in the form of sulfate (Blake-Kalff *et al.*, 1998; Wang *et al.*, submitted), which is slowly remobilized (Hawkesford and De Kok, 2006; Blake-Kalff *et al.*, 1998), S supply to the pods might be limiting during reproductive growth.

A survey of genotypic variation in S distribution to developing organs revealed significant differences among genotypes, especially between double low (DL) and glucosinolate-containing (high GSL; HG) cvs (own results). HG cvs were found superior in S remobilization to developing organs. Apart from producing more glucosinolates, HG cvs were also characterized by a higher sulfate assimilation and relatively more leaf-S in assimilated forms. For DL cvs a low S distribution to developing organs was clearly related to low sulfate assimilation. From these results it might be concluded that HG cvs should be less susceptible to S limitations in reproductive growth. On the other hand, N remobilization efficiency of HG cvs was found to be poor (own results), which might also have consequences for pod and seed set. The aim of this study was therefore to explore if genotypic variation in S distribution is related to yield and harvest index and might thus present a valuable plant trait to improve yield and N efficiency of oilseed rape. For this purpose, a set of DL and HG cvs which were found to vary in S distribution were grown in field experiments at low and high N supply, and yield as well as N and S harvest indices were investigated.

Materials and methods

Germplasm

A set of 22 oilseed rape cvs, including 13 DL cvs and 9 HG cvs, which were already characterized in short-term hydroponic experiments, were used for the field experiments. The 22 rapeseed cvs were selected from over 1000 cvs from the PreBreedYield (PBY) breeding program in order to present a broad genetic background. The 22 cvs used in this study were also chosen because of their adaptation to local growing conditions. The seeds were supplied by Norddeutsche Pflanzenzucht Lembke (NPZ, Hohenlieth, Germany).

Hydroponic experiments

Four independent hydroponic experiments were performed between October 2012 and June 2013 in the greenhouse of the Institute of Plant Nutrition and Soil Science, Kiel University, Germany. Each individual experiment lasted 62 to 66 days. The experimental layout and procedure has been described previously (Wang *et al*., submitted). Briefly, within each experiment, two plants per cv have been grown in 10 L plastic pots in nutrient solution at sufficient S (300 µM S) and low N supply (500 µM N). N supply was gradually further decreased to 100 and then 50 µM to induce leaf senescence and thus N and S remobilization of older leaves. Plants were harvested when the third true leaf counted from the base of the plants was shed. Total N and S concentrations as well as sulfate concentrations were determined for developing leaves, mature leaves, senescent leaves and the rest shoot. N (or S) distribution into developing leaves was evaluated by the ratio of N (or S) concentrations in developing and mature leaves. N remobilization efficiency (NRE) was calculated as the difference in N concentration between mature and senescent leaves divided by the N concentration in mature leaves.

Field experiments

Field experiments were performed in the growing period 2012/2013 at three locations in Germany (Reinshof, Rauischholzhausen and Rotenkirchen). Each experiment comprised two

N rates (limting N and optimal N supply) and two block replications. N was fertilized as calcium ammonium nitrate. Half of the N dose was applied at the beginning of the vegetation period and the second half at the beginning of shooting (BBCH 30; Lancashire *et al*., 1991). Two different N rates were established: no N fertilization (N0), and 240 kg N ha^{-1} (N1), with soil mineral N contents deducted from the first N dose. Pest, disease, and weed control, and all other agronomic treatments followed the recommendations for oilseed rape production in Germany.

N efficiency was defined as seed yield at limiting N supply (Graham 1984). It was sub-divided into N uptake and N utilization efficiency (Sattelmacher *et al.*, 1994). To calculate the underlying parameters for N utilization efficiency it was split up into the components N harvest index and seed N concentration (Schulte auf'm Erley *et al.*, 2011). S efficiency parameters were calculated accordingly.

Statistics

Statistical tests were done with R3.0.2. In hydroponic experiments, independent t-test was carried out to assess DL versus HG cv type differences, using individual cv means from four repeated experiments. In field experiments, N rate, cv type as well N rate × cv type interaction effects on shoot biomass, seed yield and harvest index were tested with three-way ANOVA using general linear model with N rate, cv type, N rate × cv type interaction as fixed factors and location, location × N rate, location × cv type and location × N rate × cv type interactions as random factors. Cv type comparisons were performed separately for each location and N rate by independent t-test. As the most obvious N deficiency was observed at location Reinshof, plant samples from this location were selected for N and S analysis. N rate, cv type as well N rate × cv type interaction effects on N and S efficiency parameters were analyzed by two-way ANOVA.

Results

Field experiments

Location and N rate effects on shoot biomass, seed yield and harvest index

Shoot biomass was significantly decreased at low-N supply (Figure 1a, b, c). Mean shoot dry weight at low N were 84%, 71% and 70% of the dry weights achieved at high N supply at the locations Rauischholzhausen, Reinshof and Rotenkirchen, respectively. The decrease was thus significantly lower at Rauischholzhausen than at Reinshof and Rotenkirchen. At Rotenkirchen, lower shoot dry weights were achieved than at Rauischholzhausen and Reinshof.

Seed yield was also significantly reduced by low N supply (Figure 1d, e, f). Mean seed yield at location Rauischholzhausen, Reinshof and Rotenkirchen decreased by 19%, 24% and 4%, respectively. The N rate effect was thus highest at Reinshof and almost negligible at Rotenkirchen. Considerable yield variation was also found between locations. Seed yield achieved at location Rotenkirchen was generally lower, while at Reinshof the highest seed yields were achieved.

In contrast, harvest index was significantly higher at low N supply (Figure 1g, h, i). Locations also differed in harvest index, with the highest harvest index at Reinshof (0.40), medium at Rauischholzhausen (0.32) and lowest at Rotenkirchen (0.29). The N rate effect on harvest index was more pronounced at Rotenkirchen than the other two locations, so that significant location × N rate interactions were found.

Genotypic variation in shoot biomass, seed yield and harvest index

Generally, DL cvs were superior in shoot biomass production than HG cvs (Figure 1a, b, c), but this cv type difference shifted depending on location (location × cv interaction). DL cvs had a higher shoot biomass under both N levels at Rauischholzhausen, but only under low N supply at Rotenkirchen. At Reinshof, however, no difference could be proven between DL and HG cvs at both N rates, while a high variation was found within HG cvs. Moreover, the

absence of N rate × cv interaction indicated that cv type difference in shoot biomass was similar across two contrasting N rates.

DL cvs produced a higher seed yield across N rates and locations than HG cvs, except for high N supply at location Rotenkirchen (Figure 1d, e, f). Moreover, this cv type difference was more pronounced at low N than at high N supply (N rate × cv interaction), and more pronounced at Rauischholzhausen and Reinshof than at Rotenkirchen (location × cv interaction). Apart from these two-way interactions, significant location× N rate × cv interaction was also proven true.

DL cvs were found significantly superior in harvest index than HG cvs across N rates and locations, except for at high N level at Rotenkirchen (Figure 1g, h, i). However, lack of N rate × cv and location × cv interactions indicate no shift in cv type difference across N rates and locations.

As the most obvious N deficiency was observed at the location Reinshof, plant samples from this location were selected for further analysis of N and S efficiency parameters.

Nitrogen efficiency parameters

N uptake was significantly increased at high N supply (Figure 2a). Mean N uptake at high N supply was 1.65 fold higher than at low N. DL cvs showed a higher N uptake than HG cvs at low N supply. No cv type difference was found at high N due to a high within-group variation of HG cvs. There was no N rate × cv interaction for N uptake.

In contrast, N utilization efficiency was significantly reduced by high N supply (Figure 2b). Mean N utilization at high N was 81% of that observed low N supply. Cvs differed in N utilization efficiency. DL cvs were superior in N utilization efficiency compared to HG cvs at both low and high N levels. There was no N rate × cv interaction in N utilization efficiency.

N harvest index was also significantly deceased at high N supply (Figure 2c). DL and HG cvs differed in N harvest index. Mean N harvest index of DL cvs were 13% and 38% higher than HG cvs at low and high N supply, respectively. As the N rate effect on N harvest index was

much stronger in HG cvs than that in DL cvs, a significant N rate × cv interaction effect was found.

A significant increase was observed in seed N concentration at high N supply (Figure 2d). Mean seed N concentration of DL and HG cvs were 11% and 10% higher at high N than at low N level. DL cvs had lower seed N concentrations than HG cvs at both low and high N conditions. N rate × cv interaction effect on seed N concentration was not significant, indicating the N rate effect was similar in DL and HG cvs.

Sulfur efficiency parameters

High N supply significantly improved S uptake (Figure 3a). Mean S uptake at high N was increased by 80% compared to low N. DL and HG cvs did not differ in S uptake at both N rates. No N rate × cv type interaction effect on S uptake was found.

S utilization efficiency decreased significantly at high N supply (Figure 3b). Mean S utilization efficiency at high N supply was 74% of that achieved at low N. DL cvs were much more efficient in S utilization than HG cvs. Mean S utilization efficiency of DL cvs were 1.80 and 2.06 folds of that observed with HG cvs at low and high N, respectively. This cv type difference did not shift significantly between N rates.

S harvest index was not significantly affected by N rate (Figure 3c). But HG cvs were 40% and 17% higher in S harvest index than DL cvs at low and high N rates, respectively. As the cv type difference was comparatively higher at low N than at high N condition, a significant N rate × cv interaction effect was found for S harvest index.

Seed S concentration was significantly increased by high N supply (Figure 3d). The mean seed S concentration at high N supply was 31% higher than that achieved at low N. Apart from the N rate effect, the mean seed S concentration in HG cvs were 2.58 and 2.39 fold of that observed with DL cvs at low and high N supply, respectively ($P < 0.001$). There was no significant N rate × cv type interaction.

Nitrogen and sulfur concentrations in straw

Straw N concentration at maturity increased with high N supply (Figure 4a). Mean straw N concentrations at high N supply amounted to 11.6 and 13.0 mg g^{-1} for DL and HG cvs, which were 1.29 and 1.40 fold higher than at low N supply, respectively. DL and HG cvs did not differ in straw N concentration at low N, but DL cvs had significantly lower straw N concentrations than HG cvs at high N. No N rate × cv interaction effect existed for straw N concentration.

Straw S concentration was also significantly increased by high N supply (Figure 4b). At low N mean straw S concentrations amounted to 2.96 and 2.33 mg g^{-1} for DL and HG cvs, while 3.83 and 3.14 mg g^{-1} were achieved by DL and HG cvs, respectively, at high N supply. At both N rates, DL cvs had significantly higher straw S concentrations than HG cvs. No N rate × cv interaction effect was detected for straw N concentration.

Relationships between hydroponic and field experiments

Negative relationships were found between S distribution to developing leaves ($[S]_{Dev.}$:$[S]_{Mat.}$) of HG cvs at vegetative growth and N harvest index under both low and high N supply in field experiments (Figure 5a). Genotypic variation in S distribution to developing leaves reflected 56% and 36% of the variation in N harvest index at low and high N supply, respectively. However, this relationship did not hold true for DL cvs.

Besides N harvest index, also the yield of HG cvs at both low and high N supply in the field experiments was reflected by S distribution to developing leaves at vegetative growth (Figure 5b, $R^2 = 0.59^{**}$ and $R^2 = 0.48^{**}$ for low and high N supply, respectively). S distribution to developing leaves in hydroponic experiments was closely and negatively related to yield in field experiments. (also true for other two locations: Rauischholzhausen, $R^2 = 0.37^{*}$ and $R^2 = 0.44^{*}$ for low and high N; Rotenkirchen, and $R^2 = 0.37^{*}$ and $R^2 = 0.42^{*}$ for low and high N supply, respectively). Such relationships could not be proven for DL cvs.

Discussion

The results revealed that DL cvs were superior in yield both at low and high N supply, and this was mainly due to a higher harvest index of this cv type compared to the HG cvs (Figure 1). The initially formulated expectation that HG cvs might show improved pod and seed set and therefore higher harvest indices due to their better S distribution to developing organs has therefore not been proven true. Instead, it has been found that a high S distribution to developing organs as quantified in the hydroponic experiments was clearly negatively related to yield for HG cvs (Figure 5).

One possible reason to explain this negative relationship is that a high formation of storage products in the seeds like glucosinolates and proteins is detrimental for yield. The formation of glucosinolates might consume too many C and N assimilates. There has been evidence that seed glucosinolates were not synthesized *de novo* (Bilsborrow *et al.*, 1993). Instead pod walls were considered as a major site of glucosinolate biosynthesis for seeds (Zhao *et al.*, 1993). On the other hand, pod walls are also the major organ for photosynthesis after flowering (Gammelvind *et al.*, 1996; Diepenbrock, 2000). As a consequence, the competition for assimilates for glucosinolate synthesis might depress the development of pods and seeds. It is, however, questionable if the amounts of glucosinolates synthesized are high enough to cause such an effect, at least with regard to N assimilates. The calculated maximal amount of N in glucosinolates is only 0.23% (at 0.16% S in seeds), which is far less than 10% of total N in seeds. Apart from glucosinolates, seeds of HG cvs also contained more proteins, as can be deduced from the higher seed N concentrations (Figure 2d). This is probably due to the higher sulfur assimilation in HG cvs (own results) which seems to be co-regulated with glucosinolate biosynthesis (Huseby *et al.*, 2013). The higher seed protein formation might have contributed to lower seed yield of HG cvs compared to DL cvs, but this accounted for only a minor part of the yield decrease.

Another reason for the negative relationship between sulfur distribution and yield in HG cvs might be the low N harvest index of this cv type (Figure 2), which caused a major part of the yield decrease. In the hydroponic experiments, it had been found that HG cvs generally had a lower leaf N remobilization as compared to DL cvs, leaving a higher N percentage in dead leaves (own results). The low N harvest index might therefore be caused by impaired N remobilization from the straw in HG cvs. It seems, however, that there was not generally a lower N remobilization from the straw for HG cvs. At least at low N conditions in the field experiments, the straw N concentrations were similar in HG and DL cvs (Figure 4). In addition, in contrast to S distribution, leaf N remobilization as determined in the hydroponic experiment was not correlated with N harvest index or yield in the field experiments (data not shown). It therefore seems, that the low N harvest index might rather be a consequence of the low harvest index of HG cvs, instead of being the cause of it.

It must therefore be concluded that the high S distribution to developing organs as shown by HG cvs especially enhances vegetative growth at the expense of reproductive growth or has a direct negative effect on the formation of reproductive organs, thus leading to low harvest indices and in consequence N harvest indices. This is not the case for DL cvs, for which no relationship was found between S distribution and yield (data not shown). In consequence, these effects must be due to the form of S assimilates produced by some HG cvs instead of the total amount of assimilated S, which differed generally between HG and DL cvs. In the hydroponic experiments S distribution to developing organs was clearly related to the degree of sulfate assimilation in DL cvs (own results). For HG cvs, sulfate assimilation was generally high, and the genotypic variation in S distribution for this cv group remained unclear, since it could not be related to the concentrations of glucosinolates or gluthathione in mature leaves (own results). Most probably, cvs vary in the forms of S-containing amino acids produced that were not investigated in that study. The major transport forms for S are methionine, gluthathione and S-methylmethionine (Bourgis *et al.*, 1999; Lee *et al.*, 2008; Balint and

Rengel, 2011). Gluthathione levels were similar in DL and HG cvs (own results). For S-methylmethionine positive effects of this substance on the efficiency of N translocation to seeds have been found in pea (Tan *et al.*, 2010). This is in contrast to the lower N translocation to the seeds in this study (Figure 2c). Therefore, the most promising candidate to cause the found genotypic variation might be methionine, which is also a precursor for many glucosinolates (Halkier and Gershenzon, 2006). This assumption would imply, that methionine has no positive effects on the pod or seed set in oilseed rape. Alternatively to methionine, long distance transport is also evident for glucosinolates (Halkier and Gershenzon, 2006). Therefore the possibility exists that the transport of particular glucosinolates might decrease pod or seed set and thus limit reproductive growth. Unfortunately, although a clear effect of S nutrition of pod and seed set has been proven for oilseed rape (Fismes *et al.*, 2000), it is not clear which particular S metabolites are important for these effects, or which metabolic steps related to S play a role at all for the formation of reproductive organs.

For DL cvs, no negative relationship between yield and S distribution existed, but also no positive effect of high S distribution to developing organs and harvest index or yield in field experiments was found. Since low S distribution to developing organs was related to high sulfate accumulation in this cv group (own results), it might be concluded that sufficient sulfate can be remobilized to the reproductive structures, if needed. This finding would be in accordance with previous results that verified an efficient sulfate remobilization from old leaves under S-limiting conditions, mainly due to an up-regulation of tonoplastic sulfate transporters (Dubousset *et al.*, 2009; Abdallah *et al.*, 2011).

The results of the current study show that selection for favorable S distribution in rapeseed seedlings is indeed suitable to reflect processes during reproductive growth in field experiments. However, a selection of genotypes with improved capability to remobilize S to support reproductive growth might not be necessary to improve yield capacity or the N

economy of oilseed rape, since the expected positive relationship between S distribution and reproductive growth could not be verified.

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Figure legends

Figure 1: Shoot dry weight, yield and harvest index of double low (DL, black box-plot, n = 13) and high glucosinolate cvs (HG, blue box-plot, n = 9) at low and high N supply at three locations. ANOVA results (L, location; N, nitrogen; C, cv; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant at $P < 0.05$ level) are also provided. The symbols of the box-plot indicate the following: box, 25th to 75th percentile; horizontal line, median; whiskers, 10th and 90th percentile. Stars above box-plots indicate comparison between DL and HG cvs: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant at $P < 0.05$ level, t-test.

Figure 2: N uptake (a), N utilization efficiency (NUt.E, b), N harvest index (NHI, c) and seed N concentration (d) of double low (DL, black box-plot, n = 13) and high glucosinolate cvs (HG, blue box-plot, n = 9) at low and high N supply. ANOVA results (N, nitrogen; C, cv; ***, $P < 0.001$; *, $P < 0.05$; ns, not significant at $P < 0.05$ level) are also provided. The symbols of the box-plot indicate the following: box, 25th to 75th percentile; horizontal line, median; whiskers, 10th and 90th percentile. Stars above box-plots indicate comparison between DL and HG cvs: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant at $P < 0.05$ level, t-test.

Figure 3: S uptake (a), S utilization efficiency (SUt.E, b), S harvest index (SHI, c) and seed S concentration (d) of double low (DL, black box-plot, n = 13) and high glucosinolate cvs (HG, blue box-plot, n = 9) at low and high N supply. ANOVA results (N, nitrogen; C, cv; ***, $P < 0.001$; *, $P < 0.05$; ns, not significant at $P < 0.05$ level) are also provided. The symbols of the box-plot indicate the following: box, 25th to 75th percentile; horizontal line, median; whiskers, 10th and 90th percentile. Stars above box-plots indicate comparison between DL and HG cvs: ***, $P < 0.001$; **, $P < 0.01$; ns, not significant at $P < 0.05$ level, t-test.

Figure 4: Straw N (a) and S concentration (b) of double low (DL, black box-plot, n = 13) and high glucosinolate cvs (HG, blue box-plot, n = 9) at low and high N supply. ANOVA results (N, nitrogen; C, cv; ***, $P < 0.001$; ns, not significant at $P < 0.05$ level) are also provided. The symbols of the box-plot indicate the following: box, 25th to 75th percentile; horizontal line, median; whiskers, 10th and 90th percentile. Stars above box-plots indicate comparison between DL and HG cvs: ***, $P < 0.001$; ns, not significant at $P < 0.05$ level, t-test.

Figure 5: Linear regressions between developing leaf: mature leaf S concentration ratio and N harvest index (NHI), and between developing leaf: mature leaf S concentration ratio and yield (**, $P < 0.01$; *, $P < 0.05$).

Figures

Figure 1

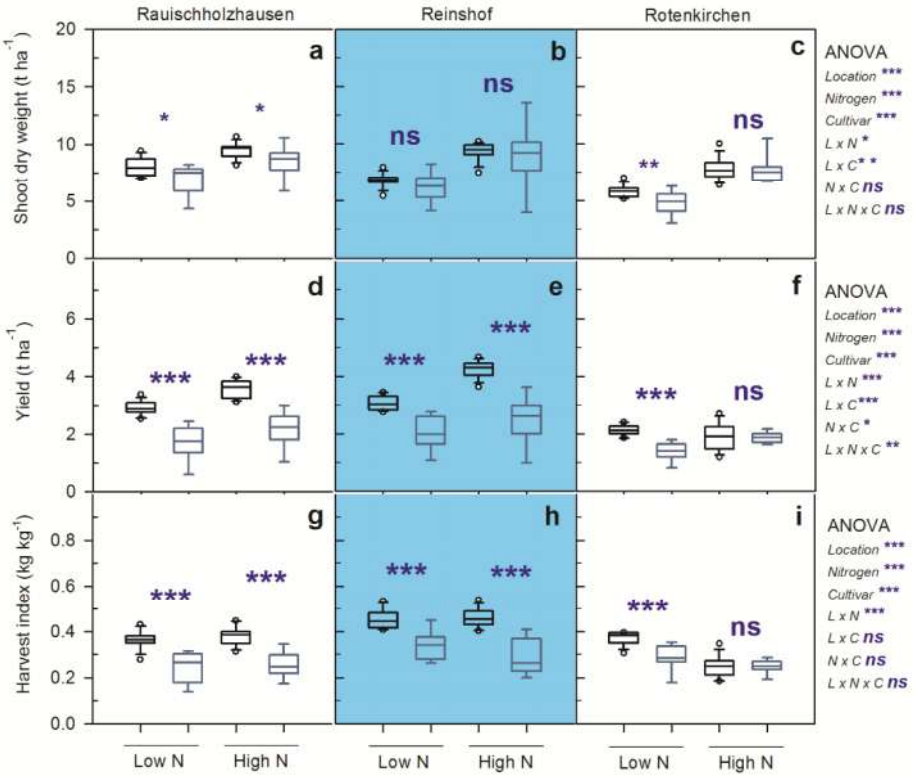
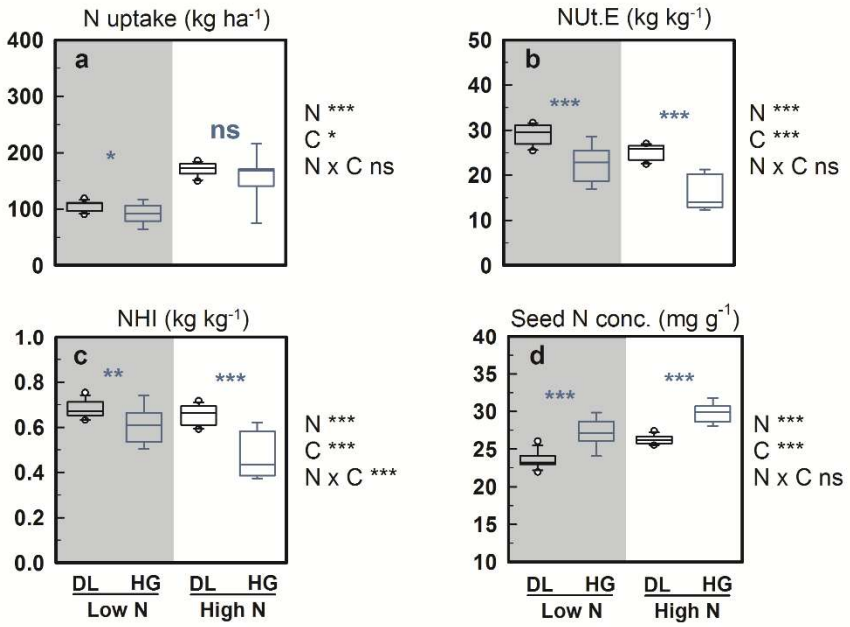


Figure 2


N uptake (kg ha-1)
a
400
300
200
100
0
*
ns
N ***
C *
N x C ns
NUt.E (kg kg-1)
b
50
40
30
20
10
0
***
***
N ***
C ***
N x C ns
NHI (kg kg-1)
c
1.0
0.8
0.6
0.4
0.2
0.0
**
***
N ***
C ***
N x C ***
Seed N conc. (mg g-1)
d
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35
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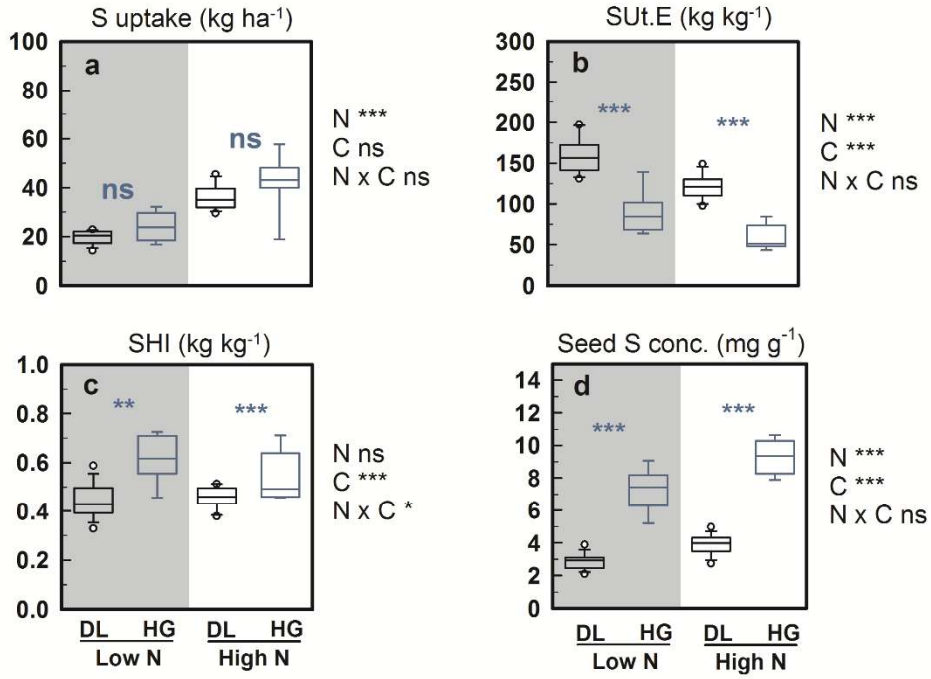
25
20
15
10
***
***
N ***
C ***
N x C ns
DL HG DL HG
Low N High N

Figure 3


S uptake (kg ha-1)
a
ns
ns
N ***
C ns
N x C ns
SUt.E (kg kg-1)
b
***
***
N ***
C ***
N x C ns
SHI (kg kg-1)
c
**
***
N ns
C ***
N x C *
Seed S conc. (mg g-1)
d
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N ***
C ***
N x C ns
DL HG DL HG
Low N High N

Figure 4

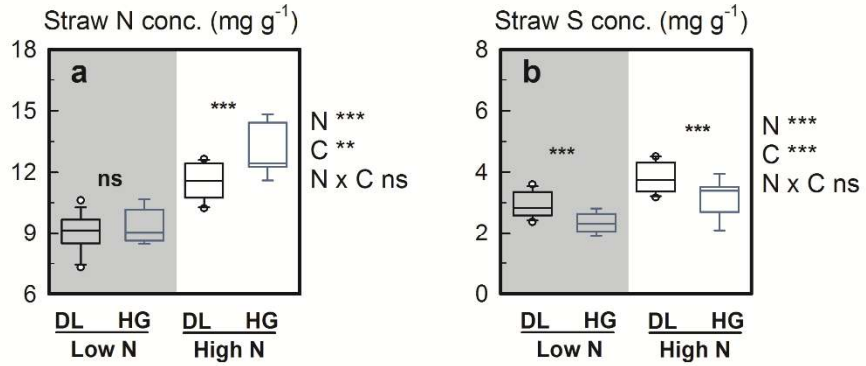
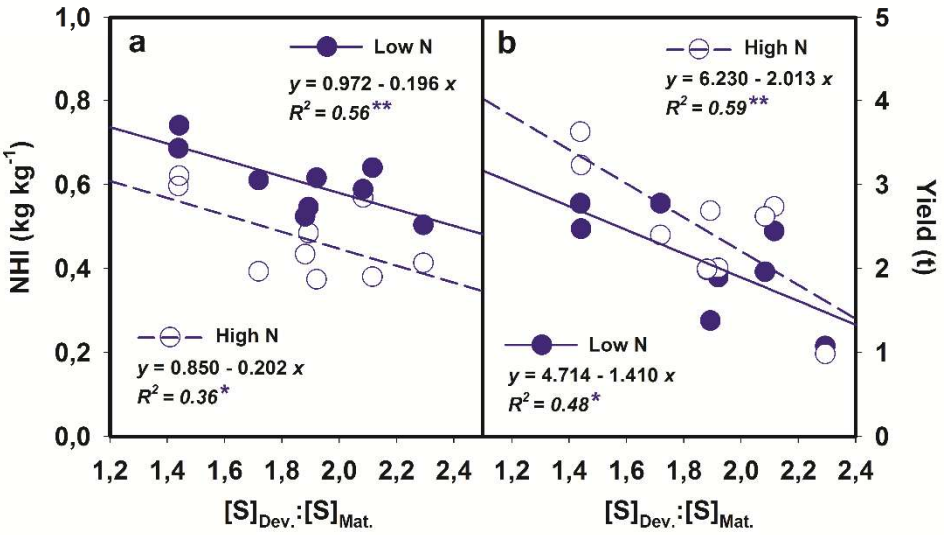

Straw N conc. (mg g-1)
18
15
12
9
6
a
ns
***
N ***
C **
N x C ns
DL HG
Low N
DL HG
High N
Straw S conc. (mg g-1)
8
6
4
2
0
b
***
***
N ***
C ***
N x C ns
DL HG
Low N
DL HG
High N

Figure 5


a
Low N
y = 0.972 - 0.196 x
R2 = 0.56**
High N
y = 0.850 - 0.202 x
R2 = 0.36*
b
High N
y = 6.230 - 2.013 x
R2 = 0.59**
Low N
y = 4.714 - 1.410 x
R2 = 0.48*
NHI (kg kg-1)
Yield (t)
[S]Dev.:[S]Mat.
[S]Dev.:[S]Mat.

Chapter 5

Genotypic variation in nitrogen efficiency and remobilization of oilseed rape cultivars differing in plant architecture

(In preparation)

Genotypic variation in nitrogen efficiency and remobilization of oilseed rape cultivars differing in plant architecture

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4 tables

4 figures

4 supplementary tables

1 supplementary figure

Key words: *Brassica napus* L.; N efficiency; remobilization; secondary branch, flowers

Abstract

Ideal plant architecture has been proposed as a mean to enhance yield potential. To better understand the impacts of plant architecture traits, such as plant height and primary/secondary branching characteristics, on N efficiency and N remobilization, a pot experiment including three oilseed rape cultivars (cv Nimrod, Asgard and Dwarf) differing in plant architecture was conducted under three N conditions (low, moderate deficient and high N supply). Genotypic variation in N efficiency traits, N remobilization from various organs as well as main stem and branching characteristics were investigated.

The results revealed that the low branching type cv Asgard was N inefficient due to poor N uptake and shoot biomass production under low N condition. Although genotypic variation in branching characteristic was also found under low N supply, but it did not seem to be a determinant for N efficiency. Two branching type cvs, i.e. Asgard and Dwarf were more N responsive because of superior N utilization efficiency resulted from higher shoot biomass and N allocation into seeds. In contrast, the Christmas tree type cv Nimrod was less so, due to more development of 'parasitic' secondary branches, and accessorial new leaves and easy-lost flowers, which depressed the biomass and N harvest indices. If N fluxes from taproot and flowers were taken into account, balance method was sufficient to estimate plant N remobilization. If not, it tended to underestimate N remobilization to seeds and lead to biased results.

It was concluded that cvs with preferential N investing in primary rather than secondary branches might be promising for improving yield potential and reducing N balance surplus especially under ample N conditions. For more precise estimation of N remobilization by balance method, N fluxes of taproot and flowers should be included in the model.

Introduction

Over the last 20 years, oilseed rape (*Brassica napus* L.) has become the second most important oleaginous crop worldwide with a 2.4-fold increase in seed production between 1992 and 2012 (Carré and Pouzet, 2014). Rapeseed oil can be used for multiple purposes (edible oil, industrial purposes, biodiesel, etc.) and the cultivation of this crop is valuable for interrupting cereal-dominated crop-rotations. A problem in oilseed rape production, however, is its high nitrogen (N) fertilizer demand that can lead to environmental N surpluses as well as detriment of the economic return (Rathke *et al.*, 2006). In a context of imposed limitations on N balance surplus by German legislation (less than 60 kg ha^{-1} in a 3-year average), the development of N-efficient cultivars (cvs) represents a priority to achieve this goal (Wiesler *et al.*, 2001; Sylvester-Bradley and Kindred, 2009).

For the selection of N-efficient genotypes in the breeding process a better knowledge of the underlying physiological mechanisms is prerequisite. N efficiency of a genotype is defined as the ability to produce a high yield under soil conditions that are N-limiting for a standard genotype (Graham, 1984). N efficient genotypes are therefore selected under N-limited conditions. The cultivation of such cvs could avoid severe yield penalties with a reduction in the N fertilization level. On the other hand, the realization of a high yield potential with increasing fertilizer rates might also play a role in reducing N balance surpluses, since high seed yield can enhance N removal from the field. Cvs that can increase yield with increasing N supply are called N responders (Gerloff, 1977). Cv performance in seed yield can shift considerably between ample and restricted N supply (Schulte auf'm Erley *et al.*, 2011). In other words, N-responsive cvs are not necessarily N-efficient and vice versa. N efficiency and responsiveness, therefore, have to be investigated separately.

N efficiency can be sub-divided into two components: N uptake and N utilization efficiency (seed dry matter yield divided by total N uptake; Moll *et al.*, 1982). While high N uptake is

highlighted as a characteristic of N efficient cvs (Berry *et al.*, 2010; Schulte auf˙m Erley *et al.*, 2011), high N utilization efficiency becomes more decisive for achieving a high yield with increasing N supply (Kessel *et al.*, 2012). N utilization efficiency depends on the N distribution in the plant and the ability to assimilate and convert CO_2 into grain carbohydrates (Sattelmacher *et al.*, 1994). Compared to wheat, rapeseed is generally efficient in converting absorbed N into biomass (Dreccer *et al.*, 2000). On the other hand, the importance of N remobilization for achieving high yields is less clear. It has been reported that more than 70 % of pod N is derived from N remobilization (Malagoli *et al.*, 2005a; Gombert *et al.*, 2010). Model calculations estimated that yield improvement of 15 % can be achieved by optimizing N remobilization from vegetative to reproductive tissues, if the additional seed N is fully converted to biomass (Malagoli *et al.*, 2005b). It might therefore be expected that cvs with better N remobilization can achieve higher yield. However, genotypic variation in yield was found not to be related to N remobilization efficiency, but rather to N uptake during reproductive growth (Schulte auf˙m Erley *et al.*, 2011; Ulas *et al.*, 2013). The importance of N remobilization vs N uptake during reproductive growth may, however, be cv-specific, since some cvs may realize their seed N allocation mainly through N remobilization with little or non-significant post-flowering N uptake (e.g. cv Capitol, Rossato *et al.*, 2001). For other cvs, however, reproductive N uptake also plays an important role in seed N filling, apart from N remobilization (Ulas *et al.*, 2013).

N remobilization efficiency and post-flowering N uptake are usually estimated from field experiments by the so-called balance method. It is based on a comparison of the total N budget of the whole plant at flowering and of both the pods (seeds) and the straw at maturity. Apparent N remobilization from the straw to the pods is then estimated as the difference between the quantity of N in the straw at flowering and that at harvest (Moll *et al.*, 1982; Ulas *et al.*, 2013). Studies of post-silking N fluxes in maize using balance method have revealed that this approach can lead to biased results for the proportion of remobilized N because it

does not take into account the contribution of the roots (Gallais *et al.*, 2006). In oilseed rape estimation based on the balance method might be even more problematic because of substantial dry matter and N losses by leaves and flowers. In this context, a global analysis of N remobilization concerning all straw organs as well as taproots seems necessary to clarify the contributions of N remobilization and post-flowering N uptake to pod N filling for different genotypes. To our knowledge such a study with different rapeseed cvs is not available yet, despite the importance.

It has long been recognized that genotypic variation in branching characteristics of oilseed rape can lead to differences in seed yield and yield components (Chauhan *et al.*, 1987). For example, increased branching has been advocated as one mean of increasing yield since the number of primary and secondary branches have a significant and positive correlation with seed yield (Katiyar and Singh, 1974; Ma *et al.*, 2014). On the other hand, an increase in branching may lead to greater retention of both dry matter and N in vegetative plant parts (Chauhan *et al.*, 1987), and thus poor harvest index and N harvest index, which are frequently yield-limiting in this crop. In particular, the low-ordered, i.e. late-flowering branches generally produce fewer flowers and pods. Also buds, flowers and pods are lost more quickly from these branches (Tayo and Morgan, 1979). As a consequence, these low-productive or non-productive branches can form 'parasitic' sinks for assimilates. In contrast, de-branching of the basal portion can induce compensatory growth in higher-order branches and thus be beneficial for total yield (Chauhan *et al.*, 1987; Tommey and Evans, 1992). In these studies, however, consequences on plant N efficiency were not described. Similar with the apical-basal sequence, secondary branches are generally later flowering than the primary ones. How far the resource investment in primary/secondary inflorescences can influence yield remains unclear. Stem is an important transient storage pool for N (Rossato *et al.*, 2001; Girondé *et al.*, 2015). In some studies, N remobilization from stems was nearly as high as that from leaves (Malagoli *et al.*, 2005a). Substantial genotypic variation in stem N remobilization has

previously been reported (Berry *et al.*, 2010), which was significantly and positively correlation with N harvest index and accounted for 71% of the variation. Cvs with higher plant height might be expected to form greater stem N pool. But the impacts of such difference on total endogenous N remobilization and N economy remain to be clarified. Therefore, three rapeseed cvs differing in branching characteristics as well as plant height were also included in the current study.

The objectives of this study were firstly to describe the variation in N efficiency of cvs differing in stem and branching characteristics, secondly to quantify and distinguish the origins of the N for pod filling in these cvs and lastly to evaluate the performance of balance method for estimating N remobilization with/without concerning flowers and taproots.

Material and methods

Experimental setup

A pot experiment was carried out between October 2012 and July 2013 at the experimental station of the Institute of Plant Nutrition and Soil Science, Kiel University, Germany. Three winter oilseed rape cvs with contrasting branching architectures were used: Cv Nimrod is a 'Christmas tree' type with a dominating main stem and many secondary branches (Figure 1). The 'low-branching' type cv Asgard produces first-order branches achieving a similar height as the main stem and less secondary branches than Nimrod, while the 'high-branching' cv Dwarf also has first-order branches with a similar height as the main stem, but originating from higher positions at the main stem, and with almost no secondary branches. Moreover, cvs differed in plant height: Nimrod is a long cv, while Asgard is a short one and Dwarf is a dwarf cv.

15 seeds were sowed in each Mitscherlich pot in October, 2012. 6 uniform plants with 4 to 6 leaves were kept over winter and thinned to 4 plants per pot thereafter. Each pot was filled with 5.8 kg dry soil containing 1.3 mg g^{-1} total N, 0.15 mg g^{-1} potassium, 0.11 mg g^{-1}

phosphorus and 2.3 % C_{org} with a pH of 6.6. Three levels of N fertilization were applied by adding 1 g (0.5, 0.25 and 0.25 g at beginning of the experiment, start of the vegetation period and beginning of shooting, i.e. BBCH 01, 16 and 30, (Lancashire *et al.*, 1991)), 2 g (0.5, 1.25 and 0.25 g at BBCH 01, 16 and 30), and 3 g N per pot (0.5, 1.25 and 1.25 g at BBCH 01, 16 and 30). Except for at BBCH 01 (0.12 g N as NH_4NO_3 and 0.38 g N as $Mg(NO_3)_2$), NH_4NO_3 was used as N source. Apart from N, each pot also received 0.60 g P (as $Ca(H_2PO_4)_2$), 2.4 g K (1.2 g as KCl, 1.2 g as K_2SO_4), 0.33 g Mg (as $Mg(NO_3)_2$), 0.50 g S (as K_2SO_4), 2 g $CaCO_3$ and micronutrients were applied (200 mg Fe, 10 mg Cu, 15 mg Zn, 30 mg Mn, 10 mg B and 2 mg Mo per pot) for optimum growth. Each N treatment was replicated 10 times for each cv and divided into two sets for harvest 1 and 2 (5 replicates each). Plants were grown under natural conditions, i.e. outside, most of the time, and were moved into a greenhouse when there was strong rain or frost. Soil moisture was kept at about 40% water capacity for seed germination, and 50% during plant growth. Fungicide Carax (0.5% v/v, BASF) was sprayed at beginning of vegetation period (BBCH 16). After that no more plant protection was applied due to absence of pests and diseases.

Harvests and measurements

For the first set, plants were harvested at flowering (BBCH 61, 21st, 18th, and 29th, April, 2013 for cv Nimrod, Asgard and Dwarf, respectively), fractionated into fully expanded leaves (16 per pot), the rest leaves, main stem, branches, flowers (buds) and taproots, oven-dried (60) and then ground into fine powder for further measurements. For the second set, 16 expanded leaves, in accordance with those in the first set, were marked at BBCH 61, and collected once they were shed. Leaves other than the marked ones were also carefully collected daily. For the purpose of collecting dead flowers, all inflorescences from one plant per pot were covered using transparent plastic bags with fine holes, which enabled obtaining all dead flowers without disturbing plant normal growth and pod forming. At maturity (BBCH 91, for cv Asgard, all plants on 2nd July; for cv Nimrod and Dwarf, low and moderate N plants on 9th

July; high N plants of cv Nimrod and Dwarf on 15th and 23rd July, respectively), the rest part of the plants were separated into main stem, branches, taproot, pod walls and seeds from main stem and primary branches, and pod walls and seeds from secondary branches. All these samples were oven-dried (60) to constant weight and finely ground for further analysis. N concentrations of the dried and ground plant fractions were determined using an elemental analyzer (Flash 2000 HT, Thermo Scientific, Germany) coupled to an isotope ratio mass spectrometer (Delta V™, Thermo Scientific, Germany).

Calculations

N content in each fraction was computed by multiplying the N concentration by dry weight.

N remobilization (flux) was calculated according to the balance method as follows:

$$N_{i,remB} = [N]_{i,flw} - [N]_{i,mtr} \quad (1)$$

where $N_{i,remB}$ was N remobilization of non-pod organ *i*. $[N]_{i,flw}$ and $[N]_{i,mtr}$ were N content in non-pod organ *i* at flowering and maturity, respectively. Correspondingly, apparent N remobilization efficiency (NRE, %) can be derived as

$$NRE_i = \frac{N_{i,remB}}{[N]_{i,flw}} \times 100\% \quad (2)$$

where NRE_i was remobilization efficiency of non-pod organ i between flowering and maturity. With balance method, the post-flowering N uptake can be calculated by the following equation:

$$N_{upB} = \sum [N]_{mtr} - \sum [N]_{flw} \quad (3)$$

where N_{upB} was post flowering N uptake by balance method, $\sum[N]_{flw}$ and $\sum[N]_{mtr}$ were the sum of N content in various organs at flowering at maturity, respectively. Assuming all N taken up post flowering was allocated into pods, pod N content by balance method was the following:

$$N_{podB} = N_{upB} + \sum N_{i,remB} \quad (4)$$

where N_{podB} was pod N content by balance method, and $\sum N_{i,remB}$ was the sum of N remobilization of all non-pod organs. The actual (measured) pod N content was derived by summing up seed N content and pod wall N content.

Statistics

Statistical tests were performed with R 3.0.2. Two-way ANOVA was used to test N rate, cv as well as their interaction effects on dry weight, N content in various organs, yield components and N efficiency parameters. To check cv difference within N rates, multiple comparisons were carried out using Tukey test in the R package 'multcomp'. To evaluate the performance of balance method, values of pod N content calculated by balance method including or excluding flower and taproot N flux were plotted against the measured values of pod N content, and the relationships were checked with simple linear regression.

Results

Seed yield, shoot biomass and harvest index

With increasing levels of N rates, both seed yield and shoot dry weight increased significantly (Figure 2A, B), and there was also a slight increase in harvest index. However, cvs differed in their response to N fertilization (N × cv interaction).

At low N supply, the superior shoot dry weight (Figure 2B) enabled cv Nimrod to achieve higher yield than cv Asgard, despite the lower harvest index (Figure 2C). Cv Dwarf also displayed significantly higher seed yield than cv Asgard due to a slightly higher (nearly significant, $P < 0.1$) shoot dry weight, but they did not differ in harvest index. It seems, therefore, shoot biomass was more decisive than harvest index for cv difference in yield with low N supply.

At moderate and high N supply, superior harvest index of the two branching cvs led to higher seed yield than cv Nimrod, even if cv Nimrod had relatively higher shoot dry weight. Therefore, higher harvest index became more important than shoot dry weight for superior yield at moderate and high N levels.

Yield components of primary branches

Higher levels of N fertilizer increased primary yield of all cvs (Table 1). This was mainly due to an increase in branch number and for the yield increase between low and moderate N supply also to an increased pod number per branch. However, apart from the general N rate effects, cvs differed in yield component responses to increasing N supply, especially because the increase in branch number with higher N supply was more distinct in the two branching cvs compared to the 'Christmas tree' type Nimrod. In addition, in cv Dwarf pod number per branch war further increased with the highest N rate, which was not the case in the other two cvs.

Apart from the differential response to increasing N supply, cvs generally differed in their yield components. Cv Dwarf was characterized by the highest primary branch number, and cv Asgard had the highest seed number per pod and the lowest 1000-seed-weight, irrespective of the N rate.

Yield components of secondary branches

Yield from secondary branches contributed between 0 to 4% to total yield at low N supply and between 1 and 24% at high N supply (Table 1). Particularly for cv Nimrod, a substantial part of the total yield was produced on secondary branches, while for cv Dwarf the contribution of secondary branches was almost nil.

Secondary yield was improved by higher levels of N fertilization (Table 1). In particular, branch number and pod number per branch of three cvs increased with enhanced N application. Also the cv variation was mainly due to differences in branch number and pod number per branch. Cv Nimrod had more secondary branches, especially at high N supply and beared more pods per branch, while these values were very low for cv Dwarf. Again, cv Asgard was characterized by high seed numbers per pod and a low 1000-seed-weight, particularly at high N.

N efficiency parameters

N uptake increased significantly with higher levels of N fertilization (Figure 2D), while N utilization efficiency decreased (Figure 2E). At low N supply Asgard was less N-efficient than the other two cvs because of inferior N uptake, but no cv variation in N utilization efficiency was proven. At moderate and high N levels, the two branching cvs were significantly higher in N utilization efficiency, but cvs did not differ in N uptake, so that the 'Christmas tree'-type cv Nimrod was non-responsive due to poor N utilization efficiency.

N utilization can be sub-divided into the two contributing factors N harvest index and seed N concentration. Increasing N supply enhanced seed N concentration, but decreased N harvest

index (Figure 2F, G). Cv differences were found in N harvest index at all N rates. At low N, cv Dwarf had a higher N harvest index than the other two cvs. At moderate N, cv Dwarf was the highest in N harvest index, followed by cv Asgard, while cv Nimrod was lowest. At high N, the two branching type cvs were higher than cv Nimrod in N harvest index. However, no genotypic variation was found in seed N concentration. Therefore, genotypic differences in N utilization efficiency resulted mainly from variations in N harvest index.

N uptake at flowering

Enhanced N fertilization significantly increased N content in all fractions at flowering (Table 2). Leaves accumulated most of the absorbed N, followed by flowers and main stem, while tap root and branches contained relatively lower proportions of total plant N.

Cvs differed in their response to N supply (N × cv interaction). At low N supply, the 'low-branching' cv Asgard had a lower total N uptake than the other two cvs mainly due to its significantly lower N content in leaves. The 'Christmas-tree' type cv Nimrod showed a higher N content in both main stem and taproots compared to the two branching cvs. 'High-branching' cv Dwarf contained less N in branches but accumulated more N in flowers than the other two cvs.

Moderate N supply changed cv ranking in total N content observed at low N supply. Cv Dwarf had a significantly higher total N content compared to the other two cvs, since it had much higher N contents in leaves and flowers. Nimrod accumulated more N in main stem and taproot than the other cvs, but had less branch N than Asgard.

At high N level, cv variation in N content of leaves and flowers remained similar as that at moderate N supply. As a consequence, cv Dwarf had significantly higher total N amount than the other two cvs. Cv Nimrod was lower in branch N content than the other two cvs. But it was still the highest in main stem and taproot N accumulation, followed by cv Dwarf, while Asgard the lowest. Therefore, Nimrod was also slightly higher than Asgard in total N content.

N content in different plant fractions at maturity

Higher N supply also increased the amount of N remaining in various straw fractions at maturity (Table 3). Dead leaves, flowers and main stem retained the majority of residual N while branches and taproot contained relatively less.

Cvs also differed in response to N levels. At low N supply, no cv difference was found in N content in dead leaves or flowers. Cv Dwarf had significantly less N remaining in main stem and branches at maturity. The much higher residual N content in taproot of cv Nimrod (133% and 60% higher than Asgard and Dwarf, respectively) led to significantly more total N retention in this cv compared to the other two.

At moderate N, cvs did not differ in N content in main stem, branches or dead flowers. Cv Dwarf had a slightly higher amount of N remaining in leaves. Cv Nimrod had significantly higher total residual N content than cv Asgard, mainly due to the 1.8-fold higher amount of N remaining in taproot.

At high N supply, cvs did not differ in N content in dead flowers and leaves. Cv Nimrod had much more N retained in main stem and branches, leading to significantly higher amount of total residual N than the other two cvs. Cv Asgard had 27 and 47% less N in main stem and taproot than cv Dwarf at maturity, but they did not differ in total remaining N content at maturity.

Apparent N remobilization efficiency

The overall N remobilization efficiency (NRE) decreased significantly with increasing N rates, resulting from substantial reductions in NRE of total leaves, branches and flowers (Table 4). Cv mean NRE of main stem did not show obvious responses to N levels, while mean NRE of taproot was enhanced at moderated and high N supply.

Cvs differed in NRE of various fractions in response to N supply (N × cv interaction). At low N, cvs did not differ in NRE of leaves or branches. Cv Dwarf was superior to the other two

cvs in total NRE mainly due to more efficient N remobilization from main stem and flowers, which present a major share of N source. Cv Asgard was more than 1-fold higher in taproot NRE than the other two cvs, since this cv had the lowest biomass but highest N concentration in the taproot at flowering (Table S1, S3). This merit, however, was not sufficient to compensate its inefficient N remobilization from main stem and flowers.

At moderate and high N supply, cv Dwarf was also superior to the other two cvs in total NRE because of its higher NRE from leaves and flowers. This was mainly due to the higher dry weight of these organs at flowering while limited cv difference was found in N concentrations in these organs. At moderate N, cv Asgard was more efficient in total N remobilization than Nimrod because it was higher in branch, flower and taproot NRE although slightly lower in main stem NRE. At high N, cv Asgard was also superior to Nimrod in total NRE due to the fact that cv Asgard became more efficient in main stem N remobilization, and that cv Nimrod displayed net N influx in branches instead of remobilization.

The calculation of NRE from fully expanded leaves allowed a more precise estimation of N remobilization efficiency processes in leaves, because this value is not influenced by the formation of new leaves. Therefore, leaf NRE from fully expanded leaves was higher than for total leaves, especially at moderate and high N supply. Cv Asgard was inferior to the other two cvs in NRE of fully expanded leaves at moderate and high N supply.

Post-flowering N uptake and seed N content

Higher N supply significantly increased both post-flowering N uptake and seed N content (Figure 3A, B), but cvs differed in the extent of their response.

At low N, cvs did not differ in post-flowering N uptake, but at moderate and high N supply cv Dwarf had a significantly lower post-flowering N uptake compared to the other two cvs.

Cv Asgard had lower seed N content than the other cvs at low N rate, which correspond to its lower yield (Figure 1A). At moderate N supply, cv Dwarf was slightly higher in seed N content than cv Nimrod. At high N, cv Nimrod had a significantly lower seed N content than the other two cvs.

Balance method evaluation

To evaluate the performance of the balance method, calculated values of pod N content by the balance method with and without considering taproot and flower N were plotted against the measured values (Figure 4A, B). The calculations of the balance method including taproot and flower N fluxes were almost perfect in predicting pod N content with only a tiny bias. However, it clearly underestimated pod N content when taproot and flower N fluxes were not included in the calculation. By means of the balance method without concerning taproot and flower N fluxes, cv ranking in predicted values of pod N content were inconsistent with the measured pod N contents. This was due to the fact that cvs differed substantially in taproot and flower N fluxes.

Discussion

Seed yield and N efficiency traits

According to the definition N efficiency is the ability of a genotype to produce a yield above average under soil conditions that are N-limiting for a standard genotype (Graham 1984), 'Christmas-tree' type cv Nimrod and 'high-branching' cv Dwarf were more N efficient than 'low-branching' cv Asgard (Figure 2 A). They might therefore enable a reduction in the N level without severe drawbacks in yield. It was reported (Schulte auf'm Erley *et al.*, 2011; Kessel *et al.*, 2012) and also verified in the present study that N uptake was more decisive than N utilization for yield at low N conditions (Figure 2 D, E). Cvs did not differ in N utilization at low N supply, while the poor N uptake led to inferior seed yield in Asgard. A second reason for the low N efficiency of Asgard was the lower shoot dry weight (Figure 2B). The lower total plant leaf area (Supplementary Figure 1) resulted in a weak shoot biomass

production in this cv, which seems to limit yield formation. However, no indication was found that N efficiency was affected by branching potential, although cv differences were found. Therefore, branching type may not present a promising trait for selecting cvs with high yield at low N. The 'long' nature of cv Nimrod resulted in a higher biomass retention in both main stem and branches (Table S4) and thus a lower harvest index (Figure 2C), but the inferior harvest index did not led to poor seed yield in this cv. It thus seems that high shoot biomass production was more important than harvest index for yield at low N.

At moderate and high N supply, the two branching type cvs significantly out-yielded the 'Christmas-tree' type cv, and were therefore more N-responsive. The higher yield in the two branching cvs was due to superior N utilization efficiency rather than N uptake (Figure 2D, E). These findings matched with earlier studies showing that N utilization efficiency was more decisive than N uptake in tracking genotypic variation in yield at high N supply (Berry *et al.*, 2010; Schulte auf'm Erley *et al.*, 2011; Kessel *et al.*, 2012). After N utilization was further sub-divided into N harvest index and seed N concentration, it was revealed that the superior N utilization of the branching cvs at moderate and high N was due to higher shoot N allocation into seeds, i.e. N harvest index. Moreover, an optimum proportion of shoot biomass allocation into seeds (i.e. harvest index), became essentially important for achieving high yield at moderate and high N levels, since shoot biomass was greatly improved for all three cvs. The results could lead to the conclusion that the lower N and biomass indices resulted in the inferior yield of cv Nimrod. The poor N and biomass distribution to seeds of Nimrod was due to higher residual N and biomass left in the longer main stem as well more secondary branches (Table 3, S4).

Origins of seed N

Generally seed N can be derived from remobilization of N taken up before the beginning of flowering and post-flowering N uptake. While Rossato *et al.* (2001) observed no post-

flowering N uptake with field-grown rapeseed, other experiments showed a significant post-flowering N uptake (Hocking *et al.*, 1997; Malagoli *et al.*, 2005a; Rathke *et al.*, 2005; Ulas *et al.*, 2013). A clear post-flowering N uptake was also found in the current study (Figure 3A). Moreover, we further revealed that the contributions of N remobilization and post-flowering N uptake to seed N varied between N rates and also between cvs, suggesting therefore different strategies in managing seed N filling.

At low N supply in the current study, a major share of the N present in seeds originated from remobilization, while only a relatively minor proportion of seed N was from post-flowering N uptake (13, 19, and 12% for cvs Nimrod, Asgard and Dwarf, respectively). These results were in line with the previous reports showing that N remobilization rather than post-flowering N uptake was the main contributor for seed N of field grown rapeseed (Ulas *et al.*, 2013). At moderate and high N levels, however, cvs Nimrod and Asgard obtained seed N mainly by reproductive N uptake (both 61% at moderate N; 84% for Nimrod and 76% for Asgard at high N), while cv Dwarf enabled a major share of seed N accumulation by N remobilization (66 and 57% at moderate and high N, respectively). This difference might be explained by the fact that cv Dwarf absorbed more N before beginning of flowering (Table 2), leading to less soil available N afterwards.

N remobilization in various organs

In agreement with previous studies (Gombert *et al.*, 2010; Ulas *et al.*, 2013; Girondé *et al.*, 2015), leaves were the main source organs for N remobilization in the present study. Leaf N remobilization of low N plants was rather efficient for all three cvs, since more than 80% of the N presented in leaves at beginning of flowering has been remobilized. Similar values were also reported in earlier studies (Ulas *et al.*, 2013). In accordance with the high remobilization efficiency, N concentration in fallen leaves in this study was much lower than that reported

previously (2-2.5 % of dry weight; Rossato *et al.*, 2001), indicating that a sink limitation may not have happened. Also no cv difference was found in residual N amount of dead leaves. Asgard had roughly 13.8 mg plant^{-1} (or 15%) less leaf N accumulation than the other cvs at beginning of flowering, leading to lower seed N content and yield. This result matched earlier studies showing a source limitation at low N supply (Gombert *et al.*, 2010; Ulas *et al.*, 2013). Therefore, our results suggested that a high leaf N content at beginning of flowering was of essential importance for optimum seed N accumulation and thus yield at low N. At moderate and high N supply, cv Dwarf was significantly higher in leaf N remobilization than the other two cvs due to much higher leaf N accumulation (+50% and +60 % at moderate and high N, respectively) before flowering (Table 2). The much higher N accumulation did not lead to a correspondingly higher residual leaf N amount (i.e. cv Dwarf had only slightly higher values at moderate N and no cv difference at high N, Table 3). Therefore, cv variation in N content of fallen leaves was not simply governed by N availability in green leaves. Instead, it can be partly explained by the fact that cv Nimrod and Asgard developed more secondary branch-attached new leaves after the beginning of flowering, which led to more leaf N retention. Growth of new leaves after flowering was also observed previously for rapeseed (Girondé *et al.*, 2015).

Stems (including branches) were described as transient storage organs in the case of an asynchronism between leaf N remobilization and N demand for seed filling (Hocking *et al.*, 1997; Rossato *et al.*, 2001; Girondé *et al.*, 2015). In order to investigate the effects of branching variation on N remobilization, main stem and branch N remobilization were evaluated separately in the current study. In agreement with previous reports (Ulas *et al.*, 2013), values of main stem NRE in this study were lower than that of leaves regardless of the N level (Table 4). Although cv Nimrod had the highest residual N content in the main stem, the much higher main stem N storage at flowering led to the highest main stem N

remobilization in this cv at all three N rates (25.6, 59.8 and 69.5 g at low, moderate and high N supply, respectively).

These results matched previous studies in rapeseed showing that the highest N remobilization was not related to a lower residual N in the stem, but to a significant higher distribution of N towards the stem before seed filling. Therefore, cvs with higher plant height to form a greater main stem N pool might be beneficial for N remobilization. It does not seem, though, that the highest N remobilization in cv Nimrod could lead to higher total N remobilization (Table 2, 3).

Branches were even more inefficient than the main stem in N remobilization, with a major part of the initial N storage at flowering time remaining as residual N (Table 4). In contrast to the main stem, branch N remobilization was not only source-governed. We further revealed clearly that it was additionally influenced by the growth of secondary branches. For example, branch N remobilization of cv Nimrod decreased dramatically with increasing N supply, and N influx (-16.3 g per plant) was observed at high N supply. This was due to the fact that enhanced N supply induced significantly more secondary branches after flowering which may represent a sink for N and increase residual N retention in total branches. Also the highest branch N remobilization of cv Asgard was observed at moderate N rather than at high N supply, since the branch N storage at moderate N reached a level slightly lower than that at high N supply (Table 2), but the residual branch N was only 51% of that at high N due to much less secondary branches (Table 1). However, the branch N remobilization of cv Dwarf increased with N levels, since there was limited growth of secondary branches and N remobilization was thus mainly source-dependent. Since the maximum genotypic variation in branch N remobilization was 2.6 and 10.6 mg per plant at low and moderate N supply, it may not contribute much to the difference in total N remobilization that can reach 24.0 and 82.5 g per plant at low and moderate N supply. At high N, since branches (mainly secondary ones) of cv Nimrod represented as sink organs that displayed net N influx instead of remobilization,

there was much less branch N remobilization in this cv compared the other two cvs. These results thus suggested that apart from lower leaf N remobilization, a much lower branch N remobilization (-28.9 mg per plant) of cv Nimrod also played a critical role for the significantly lower total N remobilization compared to cv Dwarf.

Since collection and quantification of flowers is difficult under field conditions, N remobilization from this part has long been neglected. However, importance of flowers as contributors to endogenous N remobilization could not be ruled out. In the current study, flower dry weight was much less than that of stems or leaves at flowering time, but flowers had the highest N concentrations (5.1 - 6.5% of dry mass depending on N supply, Table S1) amongst all investigated organs. Consequently flowers represent another important source organ for N remobilization in addition to leaves and stem. In accordance to the highest N concentration at flowering, flowers were also characterized by the highest residual N concentrations (2.2 - 2.8% of dry mass, Table S2). Under low N supply, flowers were rather efficient in N remobilization with a mean NRE of 72.6% (Table 4), which was higher than that of other organs except leaves. However, flower NRE deceased dramatically with increasing N supply, since higher N supply resulted in more easily-lost flowers on secondary branches.

At flowering, cv Dwarf had more flowers than the other two cvs regardless of N supply, but cvs did not differ in dry weight of dead flowers. This was due to the fact that cvs Nimrod and Asgard produced more secondary inflorescences after the first harvest at BBCH 61. Also the genotypic variation in flower NRE showed a clear response to the differences in secondary branching, i.e. more secondary inflorescences resulted in depressed NRE of cvs Nimrod and Dwarf. Taken together, these results suggested that flowers is also a crucial organ with regard to N remobilization in addition to leaves and stems. Superior flower N remobilization of cv

Dwarf partly contributed to the higher total N remobilization of this cv compared to the other two, especially under moderate and high N conditions.

Similar to stems, the taproot has also been described previously as a transient N pool (Rossato *et al.*, 2001), with N increasing during flowering and decreasing during pod filling. Vegetative storage proteins (VSP), such as 23kDa polypeptide, were suggested to be involved in these processes (Rossato *et al.*, 2001). Although VSP were not identified in the current study, substantial genotypic variation in taproot N remobilization was detected. In particular, cv Asgard had relatively lower taproot dry weight but higher N concentration at flowering (Table S1, S3), especially under low N, which led to much higher taproot NRE compared to the other two cvs. Whether differences in VSP were responsive for such cv differences in N storage and remobilization needs further investigation. However, no indication was found that the higher total N remobilization of cv Dwarf relied on a higher taproot N remobilization, since this cv had relatively lower taproot N remobilization.

Balance method

Despite the complexity of the N fluxes, several studies have been conducted to estimate globally the amount of N remobilized to the grain or seeds in maize (Gallais *et al.*, 2006; Gallais *et al.*, 2007; Coque and Gallais, 2007), rice (Sheehy *et al.*, 2004), as well as oilseed rape (Rossato *et al.*, 2001; Gombert *et al.*, 2010; Dubousset *et al.*, 2010; Ulas *et al.*, 2013), using either ^{15}N labeling or the balance method. The balance method estimated the amount of N remobilized as the difference between total plant N at flowering and total N in the straw at maturity (Moll *et al.*, 1982). This method has been found to lead to biased results in studies with maize (Coque and Gallais, 2007; Gallais *et al.*, 2007), since it neglected the contribution of roots to N remobilization and assumed that all post-anthesis N uptake was allocated to grain. In accordance with these studies, results from the current study also suggested that the balance method can cause a marked bias if N fluxes from taproots and flowers were not taken

into account (Figure 4B). Moreover, the degree of the resulting bias was cv dependent, i.e. total amount of remobilized N was more underestimated if a certain cv had more N remobilization from taproots and flowers. Consequently, it may lead to wrong conclusion in investigating genotypic variation. However, when taproot and flower N fluxes were included, the balance method worked rather perfect in estimation of N remobilization (Figure 4A). This result would imply, that the majority part of N taken up post flowering was allocated into grain whereas such newly absorbed N that allocated to straw was negligible. This will be further confirmed by subsequent investigations using the 'two labeling techniques' (i.e. ^{15}N labeling at both elongation and flowering time, (Gallais *et al.*, 2006), which could allow a precise estimation of the allocation of N taken up post flowering.

It was concluded that the 'low-branching' type cv Asgard was N inefficient due to poor N uptake and shoot biomass production under low N conditions. The two 'branching type' cvs were more N responsive because of superior N utilization efficiency resulted from higher shoot biomass and N allocation into seeds. In contrast, the 'Christmas-tree' type cv Nimrod was less N responsive due to more development of 'parasitic' secondary branches, accessorial new leaves and easily-lost flowers, which depressed the biomass and N harvest indices. Balance method taking into account N fluxes from taproot and flowers was sufficient to estimate plant N remobilization. But it tended to underestimate N remobilization to seeds and lead to biased results in genotypic variation, if the contribution from taproot and flowers was not included.

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Figure and table legends

Figure 1: Schematic view of branching characteristics of oilseed rape cultivars (MS, main stem; 1^{st} B, first order branches; 2^{nd} B, second order branches).

Figure 2: Seed yield and N efficiency traits of three rapeseed cultivars grown at three N rates. The results of F-test are given (N, N rate; Cv, cultivar; ***, $P < 0.001$; **, $P < 0.01$; ns, not significant at $P < 0.05$ level.). Cultivar differences were checked at each N rate separately. Different letters above the bars mean significantly different at $P < 0.05$ level (Tukey test). Error bar represents standard error.

Figure 3: Post-flowering N uptake (A), and seed N content (B) of three rapeseed cultivars grown at three N rates. Cultivar difference is checked at each N rate separately. Different letters above the bars mean significantly different at $P < 0.05$ level (Tukey test). Error bar represents standard error.

Figure 4: Measured pod N content in relation to calculated pod N content by the balance method including (A) or not including (B) N fluxes from taproots and dead flowers. Solid and dash lines represent the 1:1 ratio and the linear regression line, respectively. Symbols filled in white, gray and black represent values at low (1 g pot^{-1}), moderate (2 g pot^{-1}) and high (3 g pot^{-1}) N rates, respectively.

Figure S1: Fully expanded leaf area (A) and plant total leaf area (B) of three rapeseed cultivars grown at three N rates. Cultivar difference is checked at each N rate, respectively. Values were measured at the beginning of flowering. Different letters above the bars mean significantly different at $P < 0.05$ level (Tukey test). Error bar represents standard error.

Table 1: Yield components of three rapeseed cultivars grown at three N rates. Cultivar means with the same letter are not significantly different at $P < 0.05$ level within each N rate.

Table 2: N content (mg) in different plant fractions harvested at flowering of three rapeseed cultivars grown at three N rates. Cultivar means with the same letter are not significantly different at $P < 0.05$ level.

Table 3: N content (mg) in different plant fractions harvested at maturity of three rapeseed cultivars grown at three N rates. Cultivar means with the same letter are not significantly different at $P < 0.05$ level.

Table 4: Apparent N remobilization efficiency values of various plant fractions between flowering and maturity of three rapeseed cultivars grown at three N rates. Cultivar means with the same letter are not significantly different at $P < 0.05$ level.

Table S1: N concentration (%) in different plant fractions harvested at flowering time of three rapeseed cultivars grown at three N rates. Cultivar means with the same letter are not significantly different at $P < 0.05$ level.

Table S2: N concentration (%) in different plant fractions harvested at maturity of three rapeseed cultivars grown at three N rates. Cultivar means with the same letter are not significantly different at $P < 0.05$ level.

Table S3: Dry weight (g) of different plant fractions harvested at flowering time of three rapeseed cultivars grown at three N rates. Cultivar means with the same letter are not significantly different at $P < 0.05$ level.

Table S4: Dry weight (g) of different plant fractions harvested at maturity of three rapeseed cultivars grown at three N rates. Cultivar means with the same letter are not significantly different at $P < 0.05$ level.

Figures

Figure 1

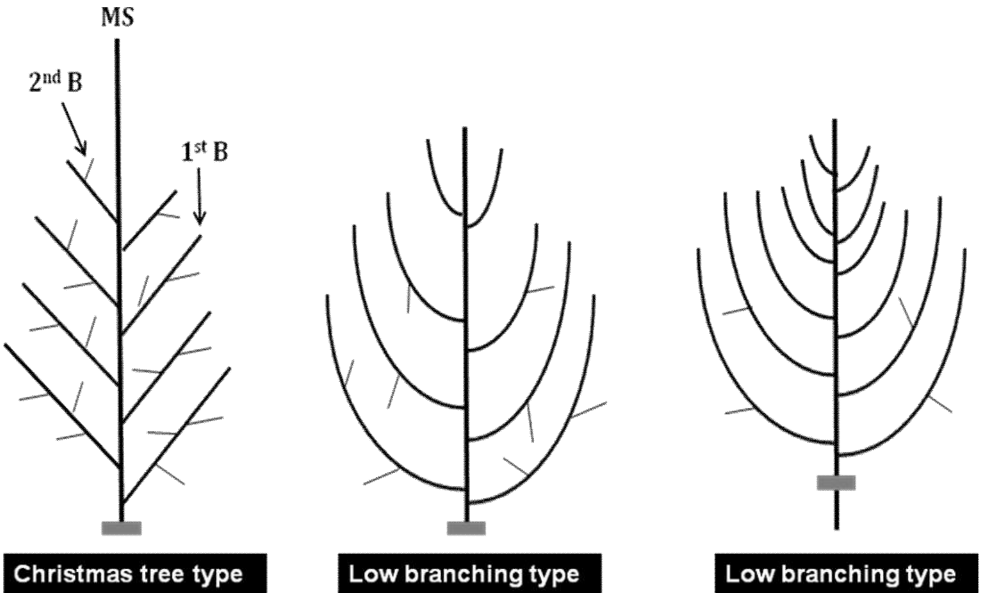


Figure 2

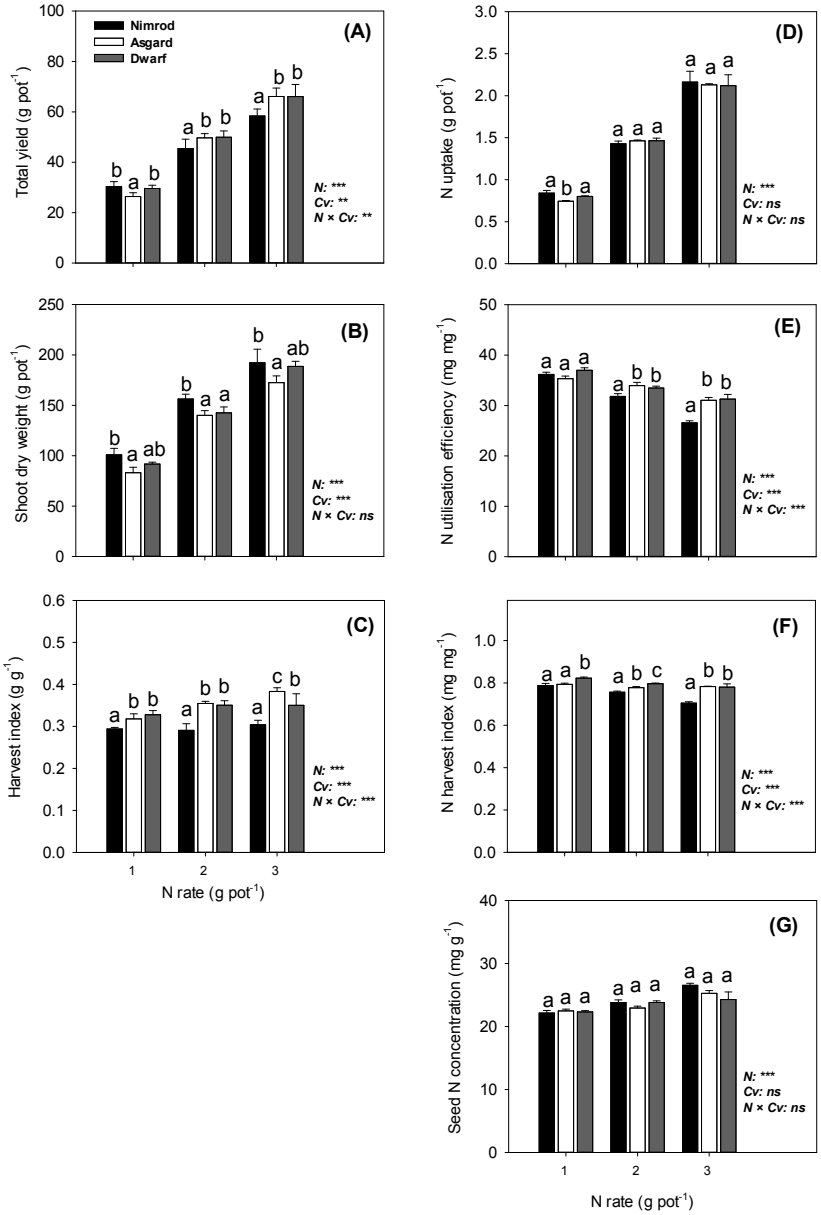

Nimrod
Asgard
Dwarf
(A)
Total yield (g pot-1)
N: ***
Cv: **
N × Cv: **
(B)
Shoot dry weight (g pot-1)
N: ***
Cv: ***
N × Cv: ns
(C)
Harvest index (g g-1)
N: ***
Cv: ***
N × Cv: ***
N rate (g pot-1)
(D)
N uptake (g pot-1)
N: ***
Cv: ns
N × Cv: ns
(E)
N utilisation efficiency (mg mg-1)
N: ***
Cv: ***
N × Cv: ***
(F)
N harvest index (mg mg-1)
N: ***
Cv: ***
N × Cv: ***
(G)
Seed N concentration (mg g-1)
N: ***
Cv: ns
N × Cv: ns
N rate (g pot-1)

Figure 3

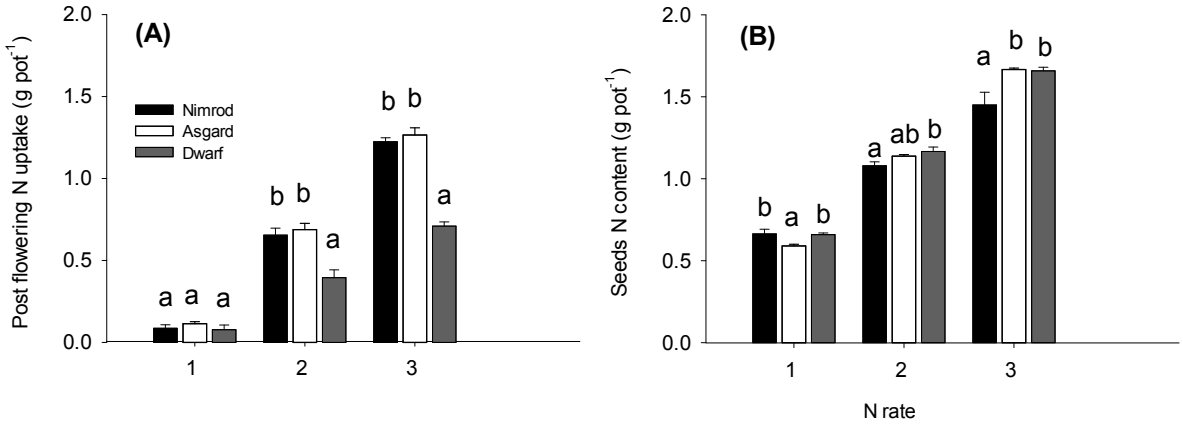

(A)
Post flowering N uptake (g pot-1)
Nimrod
Asgard
Dwarf
2.0
1.5
1.0
0.5
0.0
a a a
b b a
b b a
1
2
3
(B)
Seeds N content (g pot-1)
b a b
a ab b
a b b
N rate

Figure 4

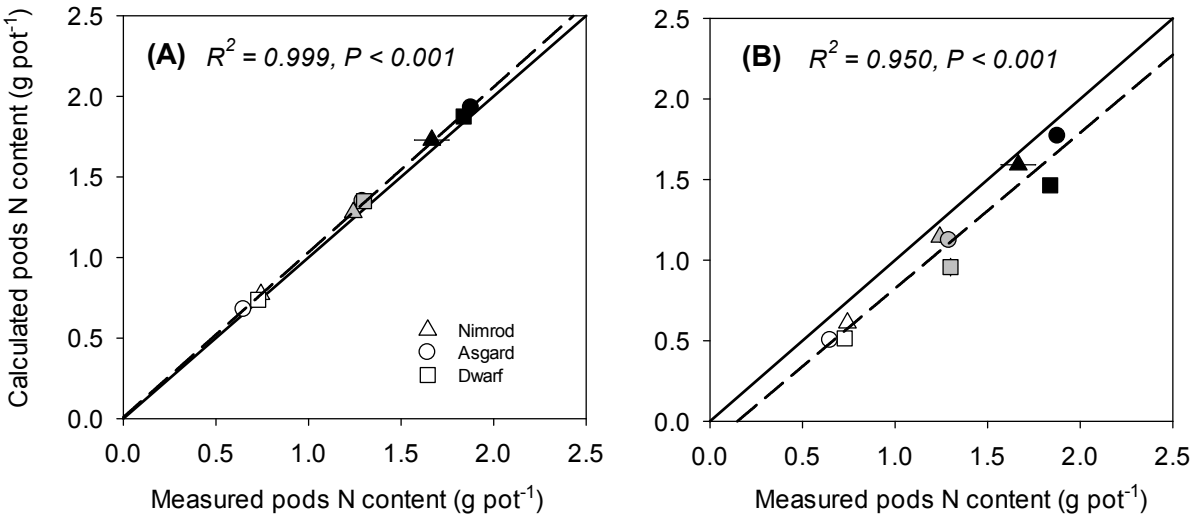
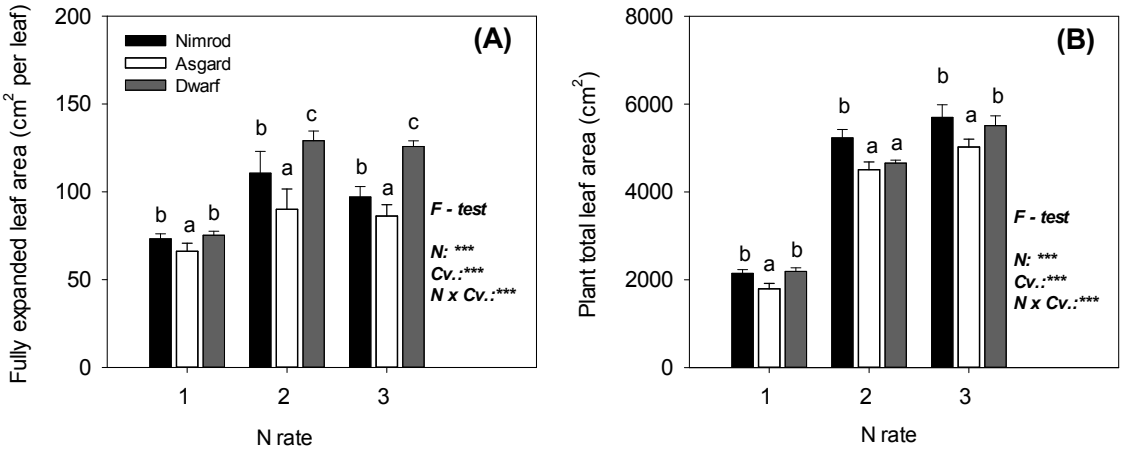

(A) R^2 = 0.999, P < 0.001
(B) R^2 = 0.950, P < 0.001
Calculated pods N content (g pot^{-1})
Measured pods N content (g pot^{-1})
Nimrod
Asgard
Dwarf
0.0
0.5
1.0
1.5
2.0
2.5

Figure S1


Fully expanded leaf area (cm2 per leaf)
200
150
100
50
0
Nimrod
Asgard
Dwarf
(A)
b
a
b
b
a
c
b
a
c
F - test
N: ***
Cv.:***
N x Cv.:***
1
2
3
N rate
Plant total leaf area (cm2)
8000
6000
4000
2000
0
(B)
b
a
b
b
a
a
b
a
b
F - test
N: ***
Cv.:***
N x Cv.:***
1
2
3
N rate

Tables

Table 1

N rate (g pot^{-1})	Component	Main stem and primary			Secondary branches		
		Nimro	Asgard	Dwarf	Nimrod	Asgard	Dwarf
1	Yield (g pot^{-1})	29.0 b	26.3 a	29.6 b	1.2 b	0.1 a	0
	Branch number (n pot^{-1})	17.0 a	18.8 a	21.8 b	5.6 a	9.2 b	0
	Pods per branch (n branch^{-}	32.4 b	25.7 a	25.4 a	4.9 b	0.5 a	-
	Seed per pod (n pod^{-1})	13.7 a	16.4 b	14.5 a	12.4 b	4.4 a	-
	1000-seed-weight (g)	3.9 b	3.4 a	3.7 b	3.6 a	3.5 a	-
2	Yield (g pot^{-1})	37.6 a	46.5 b	49.0 b	7.9 c	3.2 b	0.1 a
	Branch number (n pot^{-1})	20.0 a	20.2 a	27.4 b	21.4 b	21.8 b	5.6 a
	Pods per branch (n branch^{-}	37.9 b	38.3 b	30.9 a	8.6 c	4.2 b	0.5 a
	Seed per pod (n pod^{-1})	11.1 a	18.0 c	14.9 b	10.7 b	10.7 b	1.2 a
	1000-seed-weight (g)	4.5 c	3.4 a	3.9 b	4.0 c	3.2 a	3.6 b
3	Yield (g pot^{-1})	43.9 a	58.8 b	65.2 b	13.5 c	8.5 b	0.9 a
	Branch number (n pot^{-1})	25.2 a	23.2 a	32.8 b	41.7 c	30.8 b	19.8 a
	Pods per branch (n branch^{-}	32.4 a	36.7 a	34.6 a	8.4 c	6.5 b	1.9 a
	Seed per pod (n pod^{-1})	12.1 a	19.4 b	12.9 a	9.9 b	12.4 b	5.0 a
	1000-seed-weight (g)	4.6 b	3.6 a	4.5 b	4.9 b	3.4 a	4.7 b

Table 2

N rate (g pot^{-1})	Cultivar	Leaves		Mainstem	Branches	Flowers	Taproots	Total
		F.E.*	All					
1	Nimrod	139 a	424 b	173 b	44 b	197 a	88 b	926 b
	Asgard	133 a	368 a	144 a	40 b	221 a	59 a	833 a
	Dwarf	156 b	420 b	146 a	24 a	276 b	56 a	922 b
	Mean	142	404	154	36	231	68	894
2	Nimrod	167 a	348 a	361 b	74 a	211 a	180 b	1173 a
	Asgard	158 a	331 a	274 a	112 b	297 b	108 a	1122 a
	Dwarf	264 b	520 b	260 a	86 ab	485 c	111 a	1462 b
	Mean	196	399	299	91	331	133	1252
3	Nimrod	169 a	389 a	517 c	103 a	238 a	201 c	1448 b
	Asgard	173 a	392 a	371 a	137 b	320 b	111 a	1330 a
	Dwarf	342 b	631 b	418 b	149 b	463 c	142 b	1802 c
	Mean	228	471	435	130	340	151	1527
ANOVA	N	< 0.001	< 0.001	< 0.001	0.002	< 0.001	< 0.001	< 0.001
	Cv	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	N × Cv	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

* 16 fully expanded leave counted from the base of the plants

Table 3

N rate (g pot^{-1})	Cultivar	Leaf		Mainstem	Branches	Flowers	Taproots	Total
		F.E.*	All					
1	Nimrod	23.6 a	73.8 a	70.6 b	27.6 b	58.3 a	73.9 c	304 b
	Asgard	23.8 a	69.9 a	65.1 b	29.2 b	73.5 a	31.6 a	269 a
	Dwarf	26.9 a	81.6 a	54.9 a	17.9 a	61.4 a	46.3 b	262 a
	Mean	24.9	75.2	63.0	24.7	64.8	48.9	277
2	Nimrod	29.1 a	106 a	122 a	65.2 a	114 a	141 b	547 b
	Asgard	36.1 b	112 a	111 a	60.7 a	126 a	50.6 a	461 a
	Dwarf	42.8 c	138 b	108 a	57.3 a	143 a	59.7 a	506 ab
	Mean	36.0	119	113	61.0	128	83.7	505
3	Nimrod	23.8 a	155 a	239 c	168 b	154 a	126 b	843 b
	Asgard	35.1 b	147 a	133 a	118 a	208 a	62.2 a	669 a
	Dwarf	51.3 c	160 a	183 b	98.5 a	204 a	117 b	762 a
	Mean	35.7	154	185	130	188	100	758
ANOVA	N	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	Cv	< 0.001	< 0.001	< 0.001	< 0.001	0.020	< 0.001	0.047
	N × Cv	< 0.001	0.060	< 0.001	< 0.001	0.505	0.002	0.602

* 16 fully expanded leave counted from the base of the plants

Table 4

N rate (g pot^{-1})	Cultivar	Leaf		Mainstem	Branches	Flowers	Taproots	Total
		F.E.*	All					
1	Nimrod	82.7 a	82.6 a	59.3 ab	36.8 a	74.1 b	16.3 a	70.9 a
	Asgard	81.8 a	81.0 a	54.7 a	27.8 a	66.6 a	46.7 b	70.5 a
	Dwarf	82.7 a	80.6 a	62.4 b	26.2 a	77.3 b	20.7 a	74.3 b
	Mean	82.4	81.3	58.7	29.8	72.6	28.7	71.9
2	Nimrod	82.2 b	69.6 a	66.3 b	12.1 a	45.5 a	21.1 a	55.6 a
	Asgard	75.9 a	66.8 a	61.3 a	46.0 c	55.2 b	53.1 c	62.0 b
	Dwarf	83.8 b	73.5 b	58.5 a	33.2 b	70.0 c	45.8 b	68.2 c
	Mean	80.6	69.9	62.0	30.4	56.9	40.0	58.8
3	Nimrod	85.9 b	60.2 a	53.7 a	-62.5 a	33.8 a	40.3 b	43.4 a
	Asgard	79.6 a	62.5 a	64.1 b	13.7 b	34.3 a	43.6 b	52.2 b
	Dwarf	84.7 b	74.6 b	56.3 a	33.9 c	58.7 b	25.5 a	61.6 c
	Mean	83.3	65.1	58.2	-7.8	41.1	37.3	52.4
ANOVA	N	0.020	< 0.001	0.048	< 0.001	< 0.001	< 0.001	< 0.001
	Cv	< 0.001	< 0.001	0.885	< 0.001	< 0.001	< 0.001	< 0.001
	N × Cv	0.026	< 0.001	< 0.001	< 0.001	0.003	< 0.001	< 0.001

* 16 fully expanded leave counted from the base of the plants

Table S1

N rate (g pot^{-1})	Cultivar	Leaf		Mainstem	branches	Flowers	Taproots
		F.E.*	All				
1	Nimrod	2.38 a	3.39 b	1.11 a	2.40 a	4.93 a	1.07 b
	Asgard	2.58 b	3.47 b	1.10 a	2.67 b	5.30 b	1.22 c
	Dwarf	2.24 a	3.04 a	1.07 a	2.33 a	5.01 a	0.93 a
	Mean	2.41	3.32	1.10	2.47	5.08	1.08
2	Nimrod	2.30 a	3.03 a	1.65 b	3.38 b	5.80 a	1.56 b
	Asgard	2.57 b	3.29 b	1.62 b	3.12 b	5.85 a	1.66 b
	Dwarf	2.64 b	3.30 b	1.40 a	2.40 a	5.75 a	1.37 a
	Mean	2.50	3.21	1.56	2.97	5.80	1.53
3	Nimrod	2.70 a	3.77 a	2.66 b	4.53 a	6.43 a	2.08 b
	Asgard	3.03 b	4.25 b	2.47 a	4.37 a	6.60 a	2.10 b
	Dwarf	3.38 c	4.19 b	2.40 a	4.02 a	6.33 a	1.84 a
	Mean	3.04	4.07	2.51	4.31	6.45	2.00
ANOVA	N	< 0.001	< 0.001	0.885	< 0.001	< 0.001	< 0.001
	Cv	< 0.001	0.004	0.885	< 0.001	0.144	< 0.001
	N × Cv	< 0.001	0.004	0.064	0.005	0.839	0.866

* 16 fully expanded leave counted from the base of the plants

Table S2

N rate (g pot^{-1})	Cultivar	Leaf		Mainstem	branches	Flowers	Taproots
		F.E.*	All				
1	Nimrod	0.479 a	0.546 a	0.275 a	0.220 a	2.23 b	0.564 a
	Asgard	0.536 a	0.589 a	0.362 b	0.283 b	2.12 a	0.564 a
	Dwarf	0.519 a	0.601 a	0.264 a	0.270 b	2.30 b	0.620 a
	Mean	0.514	0.581	0.302	0.260	2.22	0.584
2	Nimrod	0.479 a	0.562 a	0.390 a	0.280 a	2.46 a	0.700 b
	Asgard	0.594 b	0.658 b	0.485 b	0.325 ab	2.35 a	0.553 a
	Dwarf	0.633 b	0.732 b	0.426 ab	0.351 b	2.47 a	0.651 ab
	Mean	0.568	0.651	0.433	0.319	2.43	0.635
3	Nimrod	0.478 a	0.685 a	0.743 b	0.475 a	2.69 a	0.638 a
	Asgard	0.573 b	0.749 b	0.576 a	0.450 a	2.83 a	0.640 a
	Dwarf	0.624 c	0.715 ab	0.645 ab	0.413 a	2.78 a	1.02 b
	Mean	0.554	0.717	0.655	0.448	2.76	0.749
ANOVA	N	0.004	< 0.001	0.885	< 0.001	< 0.001	< 0.001
	Cv	< 0.001	< 0.001	0.362	0.228	0.158	< 0.001
	N × Cv	0.078	0.031	< 0.001	0.015	0.096	< 0.001

* 16 fully expanded leave counted from the base of the plants

Table S3

N rate (g pot^{-1})	Cultivar	Leaf		Mainstem	branches	Flowers	Taproots
		F.E.*	All				
1	Nimrod	5.84 b	12.5 b	15.7 b	1.83 c	4.01 a	8.28 c
	Asgard	5.13 a	10.6 a	13.0 a	1.52 b	4.18 a	4.91 a
	Dwarf	7.11 c	14.1 c	14.4 ab	1.16 a	5.79 b	6.32 b
	Mean	5.95	12.3	14.4	1.53	4.58	6.52
2	Nimrod	7.25 a	11.5 a	21.9 b	2.21 a	3.66 a	11.7 b
	Asgard	6.14 a	10.0 a	16.9 a	3.61 b	5.11 a	6.56 a
	Dwarf	10.0 b	15.7 b	18.6 a	3.58 b	8.47 b	8.13 a
	Mean	7.81	12.4	19.1	3.13	5.75	8.79
3	Nimrod	6.25 a	10.4 b	19.5 c	2.29 a	3.69 a	9.66 c
	Asgard	5.72 a	9.19 a	15.0 a	3.15 b	4.85 b	5.33 a
	Dwarf	10.1 b	15.1 c	17.4 b	3.71 b	7.31 c	7.72 b
	Mean	7.36	11.6	17.3	3.05	5.29	7.57
ANOVA	N	< 0.001	0.005	0.885	< 0.001	0.003	< 0.001
	Cv	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	N × Cv	< 0.001	< 0.001	0.107	< 0.001	0.005	0.186

* 16 fully expanded leave counted from the base of the plants

Table S4

N rate (g pot^{-1})	Cultivar	Leaf		Mainstem	branches	Flowers	Taproots
		F.E.*	All				
1	Nimrod	4.94 b	13.5 a	25.8 c	12.5 c	2.63 a	13.2 b
	Asgard	4.42 a	11.8 a	18.1 a	10.3 b	3.48 a	5.60 a
	Dwarf	5.20 b	13.6 a	20.8 b	6.70 a	2.66 a	7.36 a
	Mean	4.85	12.9	21.2	9.66	2.94	8.39
2	Nimrod	6.08 a	18.8 a	31.3 b	23.3 c	4.66 a	20.1 b
	Asgard	6.10 a	17.1 a	22.9 a	18.8 b	5.36 a	9.18 a
	Dwarf	6.76 a	18.9 a	25.5 a	16.3 a	5.78 a	9.30 a
	Mean	6.32	18.3	26.6	19.5	5.27	12.9
3	Nimrod	4.99 a	22.6 b	32.4 c	35.6 b	5.78 a	20.1 a
	Asgard	6.14 a	19.6 a	23.1 a	26.3 a	7.35 a	9.60 b
	Dwarf	8.20 b	22.4 b	28.3 b	24.1 a	7.35 a	11.3 b
	Mean	6.32	21.5	27.9	29.0	6.79	13.8
ANOVA	N	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	Cv	< 0.001	< 0.001	< 0.001	< 0.001	0.056	< 0.001
	N × Cv	< 0.001	0.691	0.362	0.045	0.608	0.026

* 16 fully expanded leave counted from the base of the plants

Chapter 6

General discussion

6. General discussion

The first part of this study was designed to clarify genotypic variation in plant S distribution with the hypothesis that a superior S distribution to support reproductive growth is beneficial for high yield and N efficiency in oilseed rape. The second part of the current study was dealing with genotypic differences in branching characteristics and the influence on seed yield and N efficiency of oilseed rape cultivars. In this part, a global analysis of N fluxes in various organs was performed and dry matter and N investments in primary/secondary branches and the related influences on yield and N efficiency were assessed as well. The most important results and conclusions of these experiments were discussed in the following section.

6. 1. Sulfate accumulation is the crucial factor constraining S distribution into developing organs under low N conditions

The results revealed substantial genotypic variation in S distribution to developing organs (young leaves) during vegetative growth (Chapter 2, 3), especially between double low and high glucosinolate-containing (HG) cultivars. In the first investigation using four commercial rapeseed cultivars, seedlings grown at low N conditions had a higher leaf S remobilization than those grown under high N conditions (Chapter 2). This might be due to a proteolysis of insoluble S forms induced by N starvation as reported previously (Sunarpi and Anderson, 1997a; Sunarpi and Anderson, 1997b). However, sulfate contributed only a minor part of remobilized S, although most of the sulfur taken up was stored in the form of sulfate. This observation marched former studies showing that sulfate was poorly remobilized (Blake-Kalff *et al.*, 1998; Hawkesford, 2000).

Since the first hydroponic experiment using four rapeseed cultivars (Chapter 2) revealed that genotypic variation in S metabolism was expressed under low N supply only, the following hydroponic experiments were conducted under low N conditions using more cultivars, namely 13 DL and 10 HG cultivars (Chapter 3). In accordance with former studies (Schnug and

Haneklaus, 1988), no differences were found in either shoot S uptake, or mature leaf S concentration between these two groups of cultivars. But DL cultivars accumulated significantly more S in the form of sulfate than HG cultivars (89.9% vs 74.2%), which led to inferior ability in distributing S into developing leaves. Accordingly, DL cultivars had higher percentages of S in forms of GLS and insoluble-S than HG cultivars, but no indication was found that such differences resulted in the different ability in distributing S to developing organs between two groups.

It has been reported that sulfate was stored mainly in the vacuoles of mesophyll cells and was only released under conditions of prolonged S stress (Clarkson *et al.*, 1983; Bell *et al.*, 1995). In a recent study with oilseed rape, sulfate was revealed as main form of remobilized S under S-deficient conditions (Gironde *et al.*, 2014). During reproductive growth, leaves were the most important organ for S remobilization, in which sulfate remobilization was enhanced by low S availability and tonoplast SULTR4-type transporters were found specifically involved in this process. In the current study, however, no indication was found that sulfate remobilization can be improved by low N supply (Chapter 2). Sulfate accumulation was the main constraining factor leading to the difference in S distribution to developing leaves between DL and HG cultivars (Chapter 3). Previous studies coupled with our investigations may therefore lead to the conclusion that S remobilization in oilseed rape depends largely on the behavior of sulfate in plants.

In accordance with the higher S distribution to developing leaves at vegetative growth, a superior S distribution to seed, i.e. S harvest index was also observed with HG cultivars at maturity (Chapter 4). These results indicate that selection for favorable S distribution in rape seedlings is indeed suitable to reflect processes during reproductive growth.

6. 2. High S distribution into developing organs is not beneficial for reproductive growth

Although significantly higher S distribution to developing leaves at vegetative growth phase as well as higher S harvest index at maturity was observed with HG cultivars, the initial hypothesis that better S distribution into developing organs may lead to improved reproductive growth has not been proven true. Rather, higher S distribution into pods and seeds resulted in lower harvest index and yield in HG cultivars.

One possible explanation for the observed negative effects of high S distribution on harvest index and yield might be that the high formation of S-containing defense compounds like glucosinolates and S-rich proteins (Rausch and Wachter, 2005) is physiologically costly. Oilseed rape contains a unique defense system known as the glucosinolate-myrosinase system or the 'mustard oil bomb' (Ahuja *et al.*, 2011), which can be triggered by abiotic and biotic stresses, resulting in the formation of toxic products such as nitriles and isothiocyanates. However, it is traditionally viewed that such plant defense systems will lead to resource diversion, e.g. prioritization of carbon and nitrogen towards production of defense compounds at expense of plant growth (Bryant *et al.*, 1983; Fine *et al.*, 2006; Lind *et al.*, 2013).

During vegetative growth, HG cultivars were more abundant in both glucosinolates and S-rich proteins in mature leaves, which constrained leaf N remobilization to young organs to support plant growth at low N conditions (Chapter 3). During pod filling, the pod wall was the major site for photosynthesis (Zhao *et al.*, 1993). Meanwhile, it was also the major organ for glucosinolate biosynthesis for seeds (Gammelvind *et al.*, 1996; Diepenbrock, 2000). Consequently, the competition for carbon for glucosinolates synthesis depressed the development of pods and seeds in HG cultivars, which, in turn, limited pod N filling. Therefore, the results in the current study seem in support of the defense-growth-tradeoff theory.

DL cultivars generally had a better leaf N remobilization during vegetative growth, leaving a lower percentage of N in dead leaves. At maturity, these cultivars also displayed higher N removal from the field due to superior yield. Therefore DL cultivars can be suggested as N-efficient cultivars that can achieve relatively higher yield and decrease N balance surpluses. In contrast, HG cultivars were not as well suited for solving the N balance problem, but might be used as important germplasm resources for investigating the role of the glucosinolate-myrosinase system in plant-insect interactions, since many involved pathways of this defense system are still not clear and research was mainly conducted with the model plant *Arabidopsis thaliana*.

6. 3. Optimum branching is curtail for high N efficiency

It has been proven that de-branching of the low-order branches can induce compensatory growth in higher-order branches and thus increase total yield (Chauhan *et al.*, 1987; Tommey and Evans, 1992). This was due to the fact that these basal, later-flowering branches resulted in greater retention of dry matter and N (Chauhan *et al.*, 1987) in the vegetative structure. Since they were low productive or non-productive, they can therefore form 'parasitic' sinks for assimilates. However, this de-branching procedure is not practically applicable in the field. Apart from the apical-basal sequence, secondary branches are also later flowering than the primary ones. In this context, cultivars with more resources invested in primary rather than secondary branches were hypothesized to be more N-efficient. The results revealed substantial genotypic variation in primary/secondary branching characteristics at all N rates (Chapter 5). Although cultivars differed in N and biomass investment in primary/secondary branches at low N supply, a higher total N uptake and shoot biomass seem more overwhelming in determining genotypic variation in yield under these N-limiting conditions. At moderate and high N conditions, however, cultivars with more secondary branches seem to retain more residual N in these branches, which even represented a sink for N during reproductive growth. Moreover, cultivars with more secondary branches had more flower

abortion from these branches, leading to low productivity as well as low flower N remobilization. These results indicated that secondary branches may form 'parasitic' sinks for N assimilate, leading to deceased N harvest index and lack of responsiveness to N fertilizer. Therefore, our study suggested that cultivars with preferential N investing in primary rather than secondary branches might be promising for reducing N balance surpluses, especially at ample N conditions. However, it should be considered that the current study was conducted in Mitscherlich pots with a diameter of 20 cm and with only 6 plants in each pot. In the field, the planting density was usually much higher, with about 80-150 plants m^{-2} before winter and 60-80 plants m^{-2} at the beginning of spring (Ma *et al.*, 2014). High planting density results in strong competition and shading, which may further depress the development and productivity of secondary branches. Consequently, it might be expected that an optimal plant density using ideal cultivars with a preferential growth of primary branches may present a promising strategy for improving the expression of yield potential and also for deceasing N balance surplus. More experiments in the field are thus needed to clarify the interaction between population structure and genotypic variation in branching traits.

6.4. Conclusions and perspectives

Taken together, results of the current study suggest that selection for genotypes with improved ability to remobilize S to support reproductive growth is not necessary to improve yield capacity and N economy of oilseed rape. For detection of plant traits contributing to high yield and N efficiency, future efforts should focus on other nutrients, such as boron, which has specific effects on reproductive growth. Boron plays an important role in pollen germination, and pollen tube growth, and is thus crucial for pod setting. Oilseed rape requires a large B supply for plant growth and reproduction, and is highly sensitive to B deficiency (Marschner, 2012). Therefore genotypic variation in processes like boron uptake or endogenous N transport might cause differences in reproductive growth, and thus yield and N

efficiency. Genotypic variation in boron × N interaction remains largely unknown, and needs thus more investigations.

Our study shed some light on the importance of optimal plant architecture (e.g. traits of primary/secondary branches) for high yield and N efficiency. On a higher level, population structure in these traits, which is largely depending on planting density, may also influence the expression of N efficiency and yield potential. Knowledge about the interaction between planting density and genotypic variation in branching traits may therefore be helpful to achieve a high N efficiency and to solve N balance surplus problems.

Moreover, since rapeseed plants have much N loss in shed leaves before flowering while a reproductive uptake is crucial for obtaining high yield, genotypes with less vegetative N uptake but more reproductive N uptake might be suggested as N-efficient cultivars. Genotypic variation in these traits should receive more attention.

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Chapter 7

Summary/Zusammenfassung

7.1 Summary

Oilseed rape (*Brassica napus* L.) is an important agricultural crop in Europe that is characterized by the highest nitrogen (N) balance surplus as compared to other crops. Due to low reproductive N uptake and incomplete N remobilization from source organs to seeds, this crop usually leaves high amount of mineral and residual N in the field. To solve the N balance surplus problem and meanwhile to avoid severe yield penalties, a main approach is breeding and selection of cultivars that can efficiently use the available N. To facilitate the breeding process of N-efficient cultivars, many studies have been carried out to identify the secondary plant traits contributing to N efficiency, which highlighted the major importance of the reproductive growth for a high yield under conditions of low N supply, and a minor importance of vegetative growth. This thesis therefore addresses two possible aspects that are associated with plant reproductive growth, i.e. genotypic variations in sulfur distribution and in branching traits, with the aim to evaluate their impacts on N efficiency.

Objectives of the present study were (1) to determine genotypic variation in the involved S metabolites that can lead to a difference in leaf N remobilization as well as S distribution to developing organs, (2) to check if the observed genotypic variation in S distribution can be linked to yield and harvest index and thus act as a valuable plant trait to improve yield and N efficiency of oilseed rape and (3) to reveal how branching characteristics influence rapeseed yield and N efficiency. In order to investigate the impacts of genotypic variation in sulfur distribution on seed yield and N efficiency, 22 of cultivars have been grown under low N conditions in a series of hydroponic experiments, and also been evaluated under both low and high N conditions in a three-location field experiment. The influences of genotypic variation in branching traits on N efficiency were checked by a pot experiment conducted under three N rates including three oilseed rape cultivars.

In the experiments substantial genotypic variation in S distribution to developing organs was found. In particular, compared with double low cultivars high glucosinolate-containing cultivars had significantly superior ability in distributing S into developing leaves during vegetative growth, and accordingly a higher seed S harvest index at maturity. However, no indication was found that higher S distribution into reproductive organs can lead to higher yield.

Significant genotypic variations were also found in primary/secondary branching characteristics at all three N rates. At moderate and high N conditions, cultivars with more secondary branches retained more residual N in these branches. These secondary branches also had more flower abortion, leading to low productivity as well as low flower N remobilization. As a consequence, they seem to form 'parasitic' sinks for N assimilates during reproductive growth, which resulted in deceased N harvest index and lack of responsiveness to N fertilizer.

It has been concluded that selection of genotypes with improved ability to remobilize S to support reproductive growth is not necessary to improve yield capacity and N economy of oilseed rape, although selection for favorable S distribution in rape seedlings has been found indeed suitable to reflect processes during reproductive growth. Cultivars with preferential N investigation in primary rather than secondary branches might be promising for reducing N balance surpluses, especially at ample N conditions.

7.2 Zusammenfassung

Raps (*Brassica napus* L.) ist eine wichtige Kulturpflanze in Europa, die durch die im Vergleich zu anderen Kulturarten höchsten Stickstoff (N)-Bilanzüberschüsse gekennzeichnet ist. Durch die geringe reproduktive N-Aufnahme und unvollständige N-Remobilisierung aus den Source-Organen zu den Samen hinterlässt diese Kultur hohe Restmengen von mineralischem N im Feld. Um das N-Überschussproblem zu lösen und gleichzeitig deutliche Ertragsrückgänge zu vermeiden, ist ein Hauptansatz die Züchtung und Selektion von Sorten, die den verfügbaren N effektiv nutzen. Um den Züchtungsprozess N-effizienter Sorten zu vereinfachen, wurden viele Studien zur Identifikation von sekundären Pflanzeneigenschaften, die zu einer hohen N-Effizienz beitragen, durchgeführt, die die hauptsächliche Bedeutung der reproduktiven Wachstumsphase für einen hohen Ertrag unter geringer N-Versorgung hervorhoben, sowie eine untergeordnete Bedeutung der vegetativen Wachstumsphase. Diese Arbeit zielte daher auf zwei Aspekte ab, die mit dem reproduktiven Wachstum der Pflanzen im Zusammenhang stehen, und zwar die genotypische Variation in der Schwefel(S)-Verteilung und im Verzweigungsmuster, mit dem Ziel, deren Auswirkungen auf die N-Effizienz zu bewerten.

Die Ziele der vorgelegten Arbeit waren (1) die genotypische Variation in den involvierten S-Metaboliten, die zu Unterschieden in der Blatt-N-Remobilisierung und der S-Verteilung führen können, zu bestimmen, (2) zu überprüfen, ob die beobachtete genotypische Variation in der S-Verteilung mit dem Ertrag und Harvestindex im Zusammenhang steht und damit eine wertvolle pflanzliche Eigenschaft zur Verbesserung von Ertrag und N-Effizienz in Raps darstellt und (3) aufzudecken, wie Verzweigungscharakteristika Rapsertrag und N-Effizienz beeinflussen. Um die Auswirkungen der genotypischen Variabilität in der S-Verteilung auf Ertrag und N-Effizienz zu untersuchen, wurden 22 Sorten unter niedrig-N Bedingungen in einer Serie von Nährlösungsversuchen angezogen und außerdem unter niedrigen und optimalen N-Bedingungen in einem Feldversuchen an drei Standorten bewertet. Die Auswirkungen der genotypischen Variation der Verzweigungsmuster auf die N-Effizienz wurden durch einen Gefäßversuch überprüft, der unter drei N-Stufen und mit drei Rapssorten durchgeführt wurde.

In den Versuchen wurde eine substantielle genotypische Variation in der S-Verteilung in sich-entwickelnde Organe gefunden. Im Einzelnen hatten glucosinolathaltige Sorten im Vergleich zu 00-Sorten eine signifikant bessere Fähigkeit, während des vegetativen Wachstums S in sich-entwickelnde Blätter zu verteilen und hatten damit übereinstimmend einen höheren S-Harvestindex zur Reife. Allerdings wurde kein Hinweis gefunden, dass eine höhere S-Verteilung in reproduktive Organe zu einem höheren Ertrag führt.

Eine signifikante genotypische Variation wurde auch im primären/sekundären Verzweigungsmuster bei allen drei N-Stufen gefunden. Bei mittlerer und hoher N-Versorgung blieb bei Sorten mit mehr sekundären Seitentrieben mehr Rest-N in den Trieben zurück. Die sekundären Seitentriebe hatten auch einen höheren Blütenverlust, was zu einer geringen Produktivität sowie einer geringen Blüten-N-Remobilisierung führte. In Konsequenz scheinen die Triebe „parasitische“ Sinks für N-Assimilate während des reproduktiven Wachstums zu

bilden, was zu einem geringeren N-Harvestindex und einer fehlenden Reaktion auf höhere N-Düngung führt.

Es wurde gefolgert, dass die Selektion von Sorten mit einer verbesserten Fähigkeit zur S-Remobilisierung zur Erhöhung des reproduktiven Wachstums für die Verbesserung der Ertragskapazität und der N-Bilanz von Raps nicht erforderlich ist, obwohl die Selektion auf eine günstige S-Verteilung in Rapssämlingen in der Tat geeignet war, Prozesse während des reproduktiven Wachstums widerzuspiegeln. Sorten mit einer bevorzugten N-Investition in primäre statt in sekundäre Seitentriebe könnten vielversprechend für die Reduktion von N-Überschussbilanzen sein, speziell unter Bedingungen hoher N-Versorgung.

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